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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Ductal lavage is a minimally invasive method of collecting samples of milk duct cells. These cells hold promise in identifying women at increased risk of developing breast cancer and can be examined by cytopathology to determine whether they are normal, atypical or malignant. However, more sensitive molecular methods of detection have been developed. For example, aberrant methylation of genes by methylation-specific PCR (MSP) can identify tumor cells with a sensitivity of 1 in 1000 normal cells. Our objective is to analyze ductal lavage cells for aberrant methylation of genes that are frequently methylated in breast cancers but not in normal breast tissues. We have collected ductal lavage samples from 50 women. Twenty four women (48%) had breast cancer while 26 women (52%) were identified at varying degrees of increased risk for developing breast cancer based on computerized modeling. The samples were examined by cytopathology. We performed MSP analysis for 15 genes in breast cancer cell lines / primary tumors. Of these, we have identified five genes namely APC, Cyclin D2, RARβ, RASSF1A and TMS1, that are frequently methylated in tumors. Methylation analysis of these genes will be performed in breast ductal cells and will be correlated with cytological findings.

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TITLE:

Prediction of breast cancer risk by aberrant methylation in mammary duct lavage

INTRODUCTION

Breast cancer is the most common malignancy among women in the United States and is the second leading cause of cancer death in women. Invasive breast cancer originates in ductal epithelial cells and is characterized by molecular and morphologic changes that commence early in these cells. If critical early changes can be identified in breast cells, it may be possible to assign an individual's risk for development of breast cancer(1, 2). Ductal lavage is a simple noninvasive technique for collecting breast epithelial cells from the milk ducts. While cytologic examination of these cells can reveal phenotypic abnormalities, more sensitive molecular markers of disease have been developed (3). For example, aberrant promoter methylation of several genes is observed in many cancers but is rare in normal cells and is important in carcinogenesis. Methylation analysis by methylation specific PCR has the advantage that it can be performed on small amounts of DNA and can detect one malignant cell in 1000 normal cells. Our objective is to analyze breast duct cells (collected by ductal lavage) for aberrant methylation of a panel of genes that are frequently methylated in breast cancer and to correlate the results with cytologic findings to improve risk assessment for breast cancer.

BODY

The task stated under **Specific Aim #1** was to assemble a panel of genes that are frequently methylated in breast cancer. Towards this goal, we originally proposed to analyze 12 genes (APC, BRCA1, DAP Kinase, E-Cad, ER, FHIT, GSTP1, H-Cad, MGMT, p16, RARβ and TIMP-3,) reported as aberrantly methylated in cancers including genes not previously examined for methylation in breast cancer. We have collected 22 control samples, 30 breast tumor cell lines (previously established by us), 44 primary breast tissues and 28 non-malignant tissue samples for the study. The control samples were collected from healthy volunteers and consisted of peripheral blood lymphocytes (n=10) and buccal epithelial cells (n=12). Genomic DNA was extracted from all the samples and methylation analysis was performed by methylation specific PCR (MSP). One ug of DNA was treated with sodium bisulfite, which converts unmethylated cytosine

residues in CpG islands to thymines whereas methylated cytosine residues remain unchanged. Treated DNA was purified using Wizard purification kits (Promega, Madison, WI) and PCR was performed using primers specific for methylated alleles for each of the genes in the panel. PCR products were run on 2% agarose gels containing ethidium bromide and visible bands were regarded as positive. To prevent non-specific amplification, we first optimized the PCR conditions for each gene so that methylation was absent in the control samples but was present in a lymphocyte DNA sample methylated *in vitro* using Ssss1 methylase. We then performed MSP analysis on the breast cancer cell lines, primary tumors, non-malignant and control samples for these genes. In addition to the panel of genes originally proposed in the grant, we determined the methylation frequencies for the cyclin D2, TMS1 and RASSF1A genes and added these to our original panel. The methylation frequencies determined for the genes in the panel are represented in Table 1. Five genes namely APC(42), cyclin D2(45%), RARβ (27%), RASSF1A (49%), and TMS1(31%) showed frequent methylation in tumors and tumor cell lines compared to the control samples. Due to the low numbers of cells expected from the lavage samples, all or some of these five genes will be analyzed. The identification and selection of genes for the study completes the first objective of our grant proposal.

Aim #2: To determine whether the methylation profile of breast epithelial cells obtained by nipple duct lavage correlates with the degree of breast cancer risk as estimated by computerized modeling and cytological categories as determined by the Masood score (4, 5). Ductal lavage was performed on 50 women enrolled in the study. Of these, 24 women were diagnosed with cancer and 26 were considered to be at high-risk based on computerized modeling. Among the women with cancer, the racial distribution was as follows: 7 were African American, 1 was Hispanic and 16 were Caucasian whereas all 26 of the high-risk women were Caucasian. In 39 women, bilateral ductal lavge was performed while in 11 women (with cancer), only the affected breast was sampled. About 30ml of fluid was collected by ductal lavage. One half was used for cytological examination and from the other half, cells were pelleted fand stored at –70 ^{0}C for DNA extraction.

Table 1. Genes utilized for testing methylation status in cell lines breast tumors, non-malignant breast from cancer patients and control tissue from healthy volunteers and their relationship with RT-expression

Gene	Location	Function	Incidence of methylation (our data)				RT-Correlates With Methylation Cell lines	Breast Cancer Data from literature	Primer source
			Cell lines	Tumors	* Non-malignant	$ Controls			
			N= 30	N=44	N= 28	N= 22	N = 8-12		
APC	5q21-22	Binds and inactivates β-catenin	45%	42%	11%	0%	Yes	N/A	(1)
BRCA1	17q21	DNA damage repair/other	4%	-	-	0%	-	16 %	(2, 3)
Cyclin D2	12p13	Cell Cycle regulator	60%	45%	13%	0%	Yes	50%	(4)
DAP Kinase	1q21	Kinase mediator for interferon induced apoptosis	27%	-	-	9%		40%	(5)
E-Cad	16q22.1	Homotypic epithelial cell-cell adhesion molecule	5%	31%	NA	0%	Yes	48%	(6)
ER	6q25.1	Receptor for estrogen induced transcription	0%	-	-	4%	-	49%	(7)
FHIT	3p14.2	Histidine triad gene family member	86%	31%	NA	0%	Yes	N/A	(8)
GSTP1	11q13	Preventative of oxidative DNA damage	26%	-	-	0%	-	31%	(9)
H-Cad	16q24.2	Homotypic epithelial cell-cell adhesion molecule	35%	33%	6%	0%	Yes	N/A	(10)
MGMT	10q26	O^6-methylguanine-DNA-methyltransferase	0%	-	-	0%	-		(11)
p16	9p21	Cyclin dependent kinase inhibitor	13%	-	-	0%	Yes	31%	(12)
RARβ	3p24	Receptor for ligand activated transcription factor	30%	27%	11%	0%	Yes	38%	(13)
RASSF1	3p21.3	Ras binding protein	64%	49%	NA	0%	Yes	N/A	(14)
TIMP-3	22q12.3	Inhibitor of metalloproteinase	20%	-	-	4%	-	27%	(15)
TMS1	16p11-12	Target of Methylation-induced Silencing	45%	31%	3%	0%	Yes	40%	(16)

$ denotes-DNA was isolated from 12 healthy volunteers blood lymphocytes and from 10 volunteers buccal epithelium served as a negative control for standardization of the MSP-assay

* denotes -All non-malignant tissues sample are obtained from a patient with cancer.

Abbervation used: N= no of cases analysed , N/A=not available, NA= not analysed;

Blank cells with dash (-) suggest more validation analysis need to be preformed before the marker can be successfully applied to clinical specimen

Cytology reports for 37 cases have been completed. Cell counts were recorded for each sample as none, scant (<10), few(10-99), moderate (100-999), and abundant (> 1000). Cytological observations were recorded as normal, apocrine metaplasia, epithelial hyperplasia, atypia and cancer. In addition, the nuclear features were categorized and scored for parameters such as cellular arrangement, cell Pleomorphism, Myoepithelial cells, Anisonucleosis, Nucleoli, Chromatin clumping, Nuclear Diameter, Mitoses, Molding and Polarity.

Our estimate is that the number of cells retrieved by ductal lavage will vary between samples and may range from 100 to 1000 cells. Therefore, we determined the feasibility of extracting DNA, performing bisulfite treatment and subsequent purification for MSP analysis using 100 and 1000 cells from a breast cancer cell line methylated for a candidate gene (RASSF1A). MSP products were detected in both samples, indicating that the analysis can be performed using 100 cells. However, the number of target genes that can be analyzed using these low cell numbers needs to be determined. Bisulfite treated DNA for MSP analysis is not stable when subjected to freeze thaw cycles. Therefore, all assays must be performed simultaneously and within a short period after bisulfite treament.

KEY RESEARCH ACCOMPLISHEMENTS.

1. We have optimised the MSP conditions for 12 genes such that methylation is absent in peripheral blood lymphocyte and buccal epithelial samples
2. In addtion to the genes originally proposed in the grant, we analyzed the methylation status of cyclin D2, TMS1 and RASSF1A genes and we have added them to the panel.
3. We have determined the methylation frequencies for the panel of genes in breast cancer cell lines, primary breast tumors, and non-malignant samples and identified five frequently methylated genes as targets for analysis in ductal lavage samples.
4. We have collected Ductal lavage samples from 50 women and have completed cytological exams on 37 cases for cell counts as well as phenotypic characteristics such as metaplasia, epithelial hyperplasia, atypia and cancer. We have collected cell pellets from these cases for DNA extraction.

5. We have determined that MSP analysis can be performed with low cell numbers (100 cells).

REPORTABLE OUTCOME

Three manuscripts were published in collaboration with other investigator from our department, (6, 13, 17) and two manuscripts are under preparation.

CONCLUSIONS: We determined the methylation frequencies for 15 genes in breast cancer cell lines (n=30)/ primary breast tumors (n=44), and non-malignant specimens. (n=28). We identified five genes (APC, cyclin D2, RARß, RASSF1A and TMS1) that were frequently methylated in the tumors tissues but not in controls as the target genes for analysis of ductal lavage samples. Ductal lavage samples were collected from 50 women and one half of each sample was used for cytological examination. Twenty-four women were diagnosed with cancer and 26 were considered to be at high risk based on computerized modeling. Cytopathology was completed on 37 cases, and cell counts as well as phenotypic abnormalities such as atypia, metaplasia and hyperplasia were recorded. Cells were pelleted from the other half of the lavage samples for DNA extraction and were stored frozen at -70^0C. We analyzed different numbers of cells for MSP and determined that the analysis can be performed on as few as 100 cells. The methylation analysis of the target genes will be carried out on the lavage samples and results will be correlated with cytological findings.

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TITLE:
Prediction of breast cancer risk by aberrant methylation in mammary duct lavage

Abstract:

Ductal lavage is a minimally invasive method of collecting samples of milk duct cells. These cells hold promise in identifying women at increased risk of developing breast cancer and can be examined by cytopathology to determine whether they are normal, atypical or malignant. However, more sensitive molecular methods of detection have been developed. For example, aberrant methylation of genes by methylation-specific PCR (MSP) can identify tumor cells with a sensitivity of 1 in 1000 normal cells. Our objective is to analyze ductal lavage cells for aberrant methylation of genes that are frequently methylated in breast cancers but not in normal breast tissues. We have collected ductal lavage samples from 50 women. Twenty four women (48%) had breast cancer while 26 women (52%) were identified at varying degrees of increased risk for developing breast cancer based on computerized modeling. The samples were examined by cytopathology. We performed MSP analysis for 15 genes in breast cancer cell lines / primary tumors. Of these, we have identified five genes namely APC, Cyclin D2, RARβ, RASSF1A and TMS1, that are frequently methylated in tumors. Methylation analysis of these genes will be performed in breast ductal cells and will be correlated with cytological findings.

Biographical Sketches

Provide the following information for the key personnel listed on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Arvind K Virmani	Assistant Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Poona University, India	B.Sc	1973-76	Chemistry
Poona University India	M.Sc	1976-78	Biochem
University of Madras, India	M.Phil	1978-79	Biochem
University of Madras, India	Ph.D	1979-83	Biochem

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds two pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Positions Held: 1991 to date Assistant Instructor /Instructor/ Assistant Professor, Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center at Dallas, TX 75390-8593

1988 - 1991 Post doctoral fellow, Department of Pharmacy, University of Oklahoma, Health Science Center, Oklahoma city, OK 73104.

1983 - 1988 Assistant Research Officer/ Research Officer, Vector Control Research Center, (VCRC) Pondicherry, India.

Research Interests: The focus of my research is to identify molecular markers involved early in the pathogenesis of lung and breast cancers. Accumulation of mutations in genes responsible for growth control and genomic integrity lead to tumor formation. We have established a panel of lung and breast tumor cell lines and their corresponding normal lines. Using microsatellite markers and loss of heterozygosity (LOH) analysis we have identified mutational hot spots on several chromosomes spanning the entire human genome and the pattern of their sequential loss during the development of cancer. With the advent of Methylation Specific PCR (MSP) and the documentation that hypermethylation of tumor suppressor genes is important in carcinogenesis, our efforts are now aimed also at identifying hypermethylated genes as biomarkers for early diagnosis and prevention of cancer.

RESEARCH AND PROFESSIONAL EXPERIENCE (CONTINUED). PAGE LIMITATIONS APPLY.
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Selected references from a list of 57:

Young-Ah Suh, Ho-Young Lee, **Arvind Virmani**, Jiemin Wong, Koren Mann, Wilson H. Miller, Jr., Adi Gazdar, Jonathan M. Kurie Promoter Methylation and Histone H3 Hypoacetylation Contribute to Loss of Retinoic Acid Receptor-β Gene Expression in Lung Cancer..Cancer Research 62: 3945, 2002

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Aberrant Methylation of the *Adenomatous Polyposis Coli* (*APC*) Gene Promoter 1A in Breast and Lung Carcinomas[1]

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ABSTRACT

The *adenomatous polyposis coli* (*APC*) gene is a tumor suppressor gene associated with both familial and sporadic cancer. Despite high rates of allelic loss in lung and breast cancers, point mutations of the *APC* gene are infrequent in these cancer types. Aberrant methylation of the APC promoter 1A occurs in some colorectal and gastric malignancies, and we investigated whether the same mechanism occurs in lung and breast cancers. The methylation status of the *APC* gene promoter 1A was analyzed in 77 breast, 50 small cell (SCLC), and 106 non-small cell (NSCLC) lung cancer tumors and cell lines and in 68 nonmalignant tissues by methylation-specific PCR. Expression of the APC promoter 1A transcript was examined in a subset of cell lines by reverse transcription-PCR, and loss of heterozygosity at the gene locus was analyzed by the use of 12 microsatellite and polymorphic markers. Statistical tests were two-sided. Promoter 1A was methylated in 34 of 77 breast cancer tumors and cell lines (44%), in 56 of 106 NSCLC tumors and cell lines (53%), in 13 of 50 SCLC cell lines (26%), and in 3 of 68 nonmalignant samples (4%). Most cell lines tested contained the unmethylated or methylated form exclusively. In 27 cell lines tested, there was complete concordance between promoter methylation and silencing of its transcript. Demethylation with 5-aza-2′-deoxycytidine treatment restored transcript 1A expression in all eight methylated cell lines tested. Loss of heterozygosity at the *APC* locus was observed in 85% of SCLCs, 83% of NSCLCs, and 63% of breast cancer cell lines. The frequency of methylation in breast cancers increased with tumor stage and size. In summary, aberrant methylation of the 1A promoter of the *APC* gene and loss of its specific transcript is frequently present in breast and NSCLC cancers and cell lines and, to a lesser extent, in SCLC cell lines. Our findings may be of biological and clinical importance.

INTRODUCTION

The protein product of the *APC*[3] TSG at chromosome 5q21 is an important component of the Wnt signaling pathway (1), which binds to and inactivates β-catenin. Biallelic inactivation of the gene in familial adenomatous polyposis and most sporadic colorectal tumors promotes tumorigenesis (1). Inactivation of TSGs may occur via multiple mechanisms, including allelic loss, gene mutation, or by methylation of CpG sites in promoter regions. Germ-line or somatic mutations of *APC* are present in most colorectal carcinomas (2, 3), and >60% occur within the mutation cluster region, a small region of exon 15 between codons 1286 and 1513 (4). Whereas 18% of breast cancers have somatic mutations (5), mostly outside the mutation cluster region, mutations are rare or absent in other cancers, including NSCLCs (5–7). However, allelic losses at 5q21 are frequent in breast and lung carcinomas (6, 8, 9), suggesting that mechanisms other than mutation may inactivate the other allele.

Two promoters (1A and 1B) of the large *APC* gene initiate transcription from distinct sites (exons 1A and 1B), and multiple transcripts are generated by alternative splicing (10). Aberrant methylation of the 1A promoter occurs in some colorectal and gastrointestinal malignancies and is accompanied by loss of expression of its specific transcript (11–13). To determine whether this epigenetic phenomenon occurs in breast and lung cancers, we determined the methylation status of the 1A promoter in breast and lung tumors and cell lines and in nonmalignant tissues. We analyzed gene expression from this promoter in cell lines before and after treatment with a demethylating

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[3] The abbreviations used are: APC, adenomatous polyposis coli; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; Aza-CdR, 5 aza 2′-deoxycytidine; LOH, loss of heterozygosity; TSG, tumor suppressor gene.

agent. We associated our findings with allelic losses at chromosome 5q21.

MATERIALS AND METHODS

Clinical Samples. Surgically resected specimens from 42 primary breast tumors and 28 corresponding nonmalignant breast tissues from these patients were obtained from the Tumor and Tissue Repository at the Hamon Center (Dallas, TX). Tumor samples from 48 primary NSCLCs and 18 corresponding nonmalignant lung tissues were obtained from surgical resections performed at the M. D. Anderson Cancer Center (Houston, TX). For gene expression studies, six nonmalignant tissue samples (two breast, two peripheral lung tissues, and one sample each of bronchial and colonic mucosa) were obtained as far from the tumor tissue as possible. Epithelial cells from buccal swabs of 12 healthy nonsmoking volunteers and peripheral blood lymphocytes from 10 other healthy volunteers were also obtained. Appropriate Institutional Review Board permission was obtained from both participating centers, and written informed consent was obtained from all subjects. Tissues were stored at −80°C for up to 3 years before testing.

Cell Lines. Human tumor cell lines (35 breast lines, 53 SCLC lines, and 58 NSCLC lines) and B-lymphoblastoid lines (n = 47) were either established by us (14, 15) or, in a few cases, obtained from the American Type Culture Collection (Manassas, VA). Most breast and NSCLC lines were established from primary tumors, and most SCLC lines were established from metastases. Cell cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO_2.

Nucleic Acid Purification. Genomic DNA was extracted from cell pellets and tissue homogenates suspended in digestion buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 1% SDS, and 200 μg/ml proteinase K (Life Technologies, Inc.). Digestion was carried out at 50°C for 1 day, and then two extractions with phenol-chloroform (1:1; Ref. 16) were performed. Total RNA was extracted from 27 cell lines and from the six nonmalignant tissues using the Trizol Kit (Life Technologies, Inc., Rockville MD; Ref. 17).

MSP. The methylation status of the *APC* gene promoters 1A and 1B was determined by MSP (18). One μg of genomic DNA was treated with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10 mM hydroquinone (30 μl) and 3 M sodium bisulfite (pH 5.0; 520 μl; Sigma Chemical Co., St. Louis. MO) were added, and the solution was incubated at 50°C for 16 h. Treated DNA was purified by the use of a Wizard DNA purification system (Promega Corporation, Madison WI). Modified DNA was stored at −70°C until used. Bisulfite treatment converts unmethylated cytosines to uracils while leaving the methylated cytosines unaffected. PCR was performed using primer sequences essentially as described (12, 13). The primers used for amplification of the methylated form of the *APC* gene promoter 1A (13) were 5′-TATTGCGGAGTGCGGGTC-3′ (sense) and 5′-TCGACGAACTCCCGACGA-3′ (antisense); and the primers used for amplification of the unmethylated form of the APC promoter 1A were 5′-GTGTTTTATTGTGGAGTGTGGGTT-3′ (sense) and 5′-CCAATCAACAAACTCCCAACAA-3′ (antisense). Amplifications of 35 cycles were performed using HotStarTaq DNA Polymerase (Qiagen Inc., Valencia, CA) at annealing temperatures of 64°C (for the primer pair that detects the methylated sequence) and 62°C (for the primer pair that detects the unmethylated sequence). PCR products were analyzed on 2% agarose gels.

A positive control for each MSP reaction was provided by the use of normal lymphocyte DNA that had been methylated by treatment with Sss I DNA methyltransferase (New England Biolabs, Inc., Beverly, MA) before bisulfite modification (19). A water blank was used as a negative control in each set of PCR reactions. To confirm methylation status, MSP products of six cell lines were purified on agarose gels, precipitated with ethanol, and sequenced (PE Biosystems Model 377; Norwalk, CT) using big dye chemistry.

RT-PCR. APC transcripts from exons 1A, 1B, and 6–10 were analyzed by RT-PCR of RNA from 27 tumor cell lines (16 breast lines and 11 lung lines) and from 6 nonmalignant tissue samples (2 breast, 1 bronchial mucosa, 2 peripheral lung, and 1 colonic mucosa). One μg of total RNA treated with 1 unit of DNase I (Life Technologies, Inc.) was reverse-transcribed into DNA using SuperScript II First Strand Synthesis System (Life Technologies, Inc.) at 42°C for 52 min using oligo-dT primer according to the manufacturer's instructions. The resulting cDNA was then subjected to PCR using the conditions and primers for exons 1A, 1B, and 6–10 as described (12). To confirm the integrity of the RNA preparation, RT-PCR was performed using primers for the housekeeping gene β-*actin* (12).

Eight of the 27 tumor cell lines (four each of lung and breast) in which the APC promoter 1A had been demonstrated to be methylated were treated with the demethylating agent Aza-CdR. One million cells were seeded into T175 flasks and incubated in culture medium with and without Aza-CdR (2 μg/ml) for 7 days with culture medium changes on days 1, 3, and 5 (20). Cells were harvested at the end of day 7 for RNA extraction and RT-PCR analysis.

Analysis of LOH. Ten microsatellite markers flanking the *APC* gene on chromosome 5q21 analyzed are D5S656, D5S658, D5S489, D5S346, D5S1468, D5S404, D5S494, D5S639, D4S429, D5S471, and two restriction fragment-length polymorphic markers within exons 11 and 15 of the *APC* gene (21) were used for LOH analysis. DNA from 47 paired tumor and B-lymphoblastoid cell lines (13 SCLC, 18 NSCLC, and 16 breast) were analyzed for LOH as described previously (22). Briefly, 20 ng of genomic DNA were amplified by PCR in the presence of ^{32}P-α-labeled deoxycytidine-5′-triphosphate using primers for the microsatellite markers. The PCR products were separated by electrophoresis in 6% polyacrylamide gels containing 7 M urea and visualized by autoradiography. The polymorphic markers in exons 11 and 15 were analyzed in the same way, except that the PCR products were separated on a nondenaturing 6% polyacrylamide gel using the conditions for single-strand conformational polymorphism analysis and not by restriction analysis (23). Markers that amplified two distinguishable bands of different size but similar intensity in the lane having B-lymphoblastoid (constitutional) DNA were termed informative (*i.e.*, heterozygous). LOH (in tumor cell line DNA) was defined

Table 1 Frequency of methylation of the *APC* gene promoter 1A in breast and lung cancers, cancer cell lines, and control tissues

The differences in methylation frequencies between SCLC and NSCLC lines were significant (two-sided $P = 0.001$; Fisher's exact test) for all samples.

Samples	No. tested	No. methylated (%)[a]
Breast carcinoma		
Primary breast cancers	42	19 (45)
Breast cancer cell lines	35	15 (42)
Total breast cancer samples	77	34 (44)
SCLC		
Cell lines	50	13 (26)
NSCLC		
Primary tumors	48	22 (46)
Cell lines[a]	58	34 (59)
Total NSCLC samples	106	56 (53)
Nonmalignant tissues		
Peripheral blood lymphocytes[b]	10	0 (0)
Buccal swabs[b]	12	0 (0)
Nonmalignant breast[c]	28	3 (11)
Nonmalignant peripheral lung[c]	18	0 (0)
Total nonmalignant samples	68	3 (4)

[a] The *APC* gene methylation frequency by tumor subtype was 72% (18 of 25) in adenocarcinomas, 50% (3 of 6) in squamous cell carcinomas, and 48% (13 of 27) in other subtypes (large cells, carcinoids, and mixed tumors).

[b] From healthy volunteers.

[c] From resections for breast and lung cancers.

as the loss of a band corresponding to one of the two alleles present in informative cases.

Statistical Analysis. Statistical differences between groups were examined by using Fisher's exact test with continuity correction. The association of methylation frequency with clinicopathological features was analyzed using the Mantel-Haenszel χ^2 test, two-sided. Ps < 0.05 were considered statistically significant.

RESULTS

Methylation of the *APC* Gene Promoters 1A and 1B in Breast and Lung Cancers. We tested breast and lung cancer cell lines and tumors and adjacent tissues for APC promoter methylation (Table 1). The first 47 malignant breast and lung samples tested for promoter 1B methylation were negative, and additional studies were limited to promoter 1A. (13). APC promoter 1A was methylated in 44% of breast and 53% of NSCLC tumors and tumor cell lines analyzed and in 26% of the SCLC cell lines. Frequencies of methylation in NSCLC tumors and cell lines were statistically significantly higher than in SCLC cell lines ($P = 0.001$). The methylation frequencies of breast or NSCLC tumors and cell lines were similar. APC promoter 1A was not methylated in lymphocytes or epithelial cells from healthy volunteers, or in nonmalignant peripheral lung tissues. A low frequency of methylation (11%) was seen in nonmalignant breast tissues. Representative examples of the MSP products of bisulfite-treated samples using primers for specific unmethylated and methylated sequences are shown in Fig. 1*A* (see legend to Fig. 1 for details).

The unmethylated amplicon product of promoter 1A was always present in tumor tissues, whether or not the methylated form was present. However, 91% of tumor cell lines had either the methylated or unmethylated APC promoter 1A sequences, whereas 9% had both. Tumor specimens always contain nonmalignant cells, presumably the source of the unmethylated sequences in methylated tumor samples.

To confirm the findings, the laboratories of Adi F. Gazdar (UT Southwestern Medical Center, Dallas, TX) and James Herman (The Johns Hopkins Oncology Center, Baltimore, MD) exchanged 3 DNA samples from lung and breast carcinoma cell lines. The samples were analyzed in a blinded fashion in both laboratories using the same primer pairs, and similar PCR steps. The methylation-positive rates from the two laboratories were 11 of 23 (48%) and 12 of 23 (52%), with a concordant rate of 20 of 23 (87%).

Direct sequencing of methylated DNA PCR products from six cell lines (breast and NSCLC) confirmed that all cytosines not at CpG sites were converted to uracils by bisulfite treatment, whereas the cytosines at all seven CpG sites remained unchanged.

APC *Gene* Expression in Breast and Lung Cancer Cell Lines. We studied 27 of the tumor cell lines for gene expression of specific transcripts from exons 1A and 1B as well as from exons 6–10 (which are spliced transcripts originating from exons 1A or 1B). The exon 1A transcript was not expressed in any of the 8 cell lines (4 breast, 3 NSCLC, and 1 SCLC) in which the promoter was methylated, but it was expressed in all 19 unmethylated cell lines (12 breast and 7 NSCLC). Thus, expression of the exon 1A transcript showed complete concordance with the methylation status of its promoter. Of the eight methylated cell lines, three also contained an unmethylated allele. All of these three lacked expression. Presumably, heterogeneous amounts of promoter methylation in these lines significantly reduces expression, or the unmethylated allele is silenced by another mechanism. In contrast, transcripts from exons 1B and 6–10 were expressed in all cell lines tested. Transcripts from exons 1A and 1B were present in all nonmalignant tissues examined (two breast, two peripheral lung, and one each of bronchial and colonic mucosa). Treatment with the demethylating agent Aza-CdR of all eight methylated cell lines tested restored expression of transcript 1A. Fig. 1*B* shows representative examples of RT-PCR analysis of *APC* gene expression using primers corresponding to exon 1A, exon 1B, and exons 6–10.

LOH Analysis of Tumor Cell Lines. Of the 143 breast and lung cancer cell lines studied for methylation status, paired B-lymphoblastoid cell lines were available from 47. These 47 lines were tested for LOH at 5q21–22 using 12 polymorphic markers (Table 2). They included 21 of the 27 lines examined for gene expression. LOH involving one or more markers was present in 10 of 15 (67%) methylated cell lines and in 27 of 32 (84%) unmethylated cell lines. The differences between methylated and unmethylated cell lines were not significant.

Clinical Associations. Frequencies of methylation in breast tumors and cell lines were similar and therefore their combined results were correlated with patient age, race, or nodal status and with tumor histology, size, or stage. The frequency of promoter 1A methylation in breast cancers was associated with increased tumor stage ($P = 0.01$) and tumor size ($P = 0.05$;

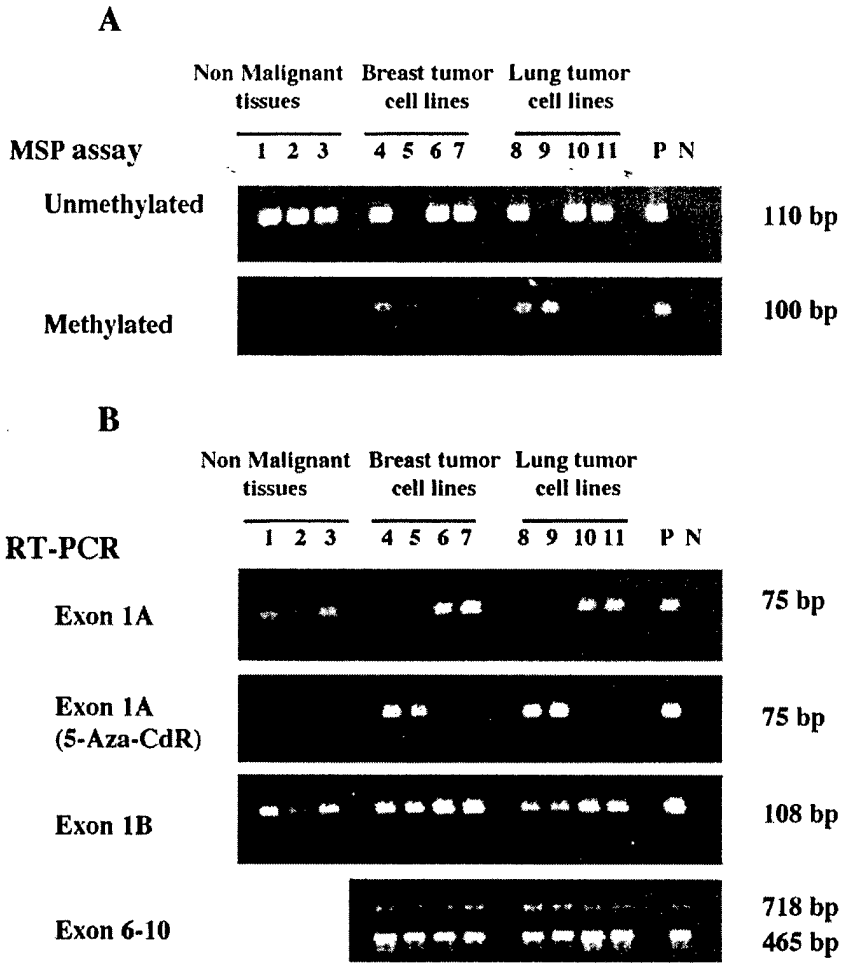

Fig. 1 MSP and expression analysis of the *APC* gene in lung and breast tumor cell lines. *Lanes 1, 2,* and *3,* nonmalignant breast tissue, peripheral lung, and bronchial mucosa, respectively; *Lanes 4–7,* breast tumor cell lines (HCC712, HCC1954, HCC38, and HCC1395, respectively); *Lanes 8–11,* lung tumor cell lines (HCC44, H2126, H2009, and H1607, respectively). *A,* the PCR products of APC promoter 1A, 110-bp unmethylated form (*top*) and 100-bp methylated form (*bottom*). Positive control (*P*) is normal lymphocyte DNA (for unmethylated form) or normal lymphocyte DNA treated with Sss1 methyl transferase (for methylated form); negative control (*N*) is water blank. *B,* RT-PCR analysis of *APC* gene expression using primers corresponding to exons 1A (*top gel*), 1B (*third gel*), and 6–10 (*bottom gel*; samples 1–3 were not analyzed for exons 6–10). Only samples 4, 5, 8, and 9, which were negative for transcript of exon 1A, were treated with the demethylating agent Aza-CdR and resulted in reexpression of transcript from exon 1A (*second gel*). Positive control (*P*) is peripheral lung DNA; negative control (*N*) is water blank.

Table 2 LOH at chromosome locus *5p21* in breast and lung cancer cell lines

		No. with LOH / No. of informative cases (% LOH)			
Marker[a]	Marker type	Breast (n = 14)	SCLC (n = 13)	NSCLC (n = 18)	P^b
D5S656	Microsatellite	4/6 (67)	4/7 (57)	1/10 (10)	0.1
D5S658	Microsatellite	8/11 (73)	6/8 (75)	3/12 (25)	0.07
D5S489	Microsatellite	4/9 (44)	5/6 (83)	1/10 (10)	**0.01**
D5S346	Microsatellite	10/12 (83)	8/10 (80)	3/12 (25)	**0.03**
APC exon 11	RsaI	3/5 (60)	6/6 (100)	2/5 (40)	0.07
APC exon 15	AspHI	5/7 (71)	6/6 (100)	2/5 (40)	0.07
D5S1468	Microsatellite	5/8 (62)	3/6 (50)	2/6 (33)	1.0
D5S404	Microsatellite	7/10 (70)	7/10 (70)	4/12 (33)	0.19
D5S494	Microsatellite	7/9 (78)	7/12 (80)	2/12 (17)	0.09
D3S639	Microsatellite	7/10 (70)	6/8 (75)	2/3 (67)	1.0
D5S429	Microsatellite	8/15 (53)	7/7 (100)	2/12 (17)	**0.01**
D5S471	Microsatellite	6/8 (75)	7/9 (78)	5/12 (42)	0.18
Any marker		11/16 (69)	11/13 (85)	15/18 (83)	

[a] Although the precise order of the markers is controversial, the markers are arranged, as best we could determine, in order from centromeric (D5S656) to telomeric (D5S471).

[b] Comparison of LOH frequencies between SCLC and NSCLC lines was performed using Fisher's exact test, two-sided. Statistically significant values are shown in boldface.

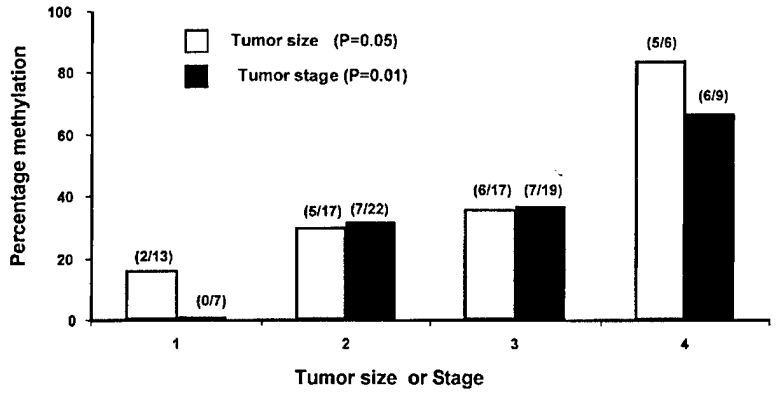

Fig. 2 Relationship between APC promoter 1A methylation of breast tumors and cell lines with breast tumor size and stage. Tumor size and stage categories were assigned using the criteria of the American Joint Committee on Cancer (43). There is a statistically significant trend of increasing methylation with tumor size ($P = 0.05$) and with tumor stage ($P = 0.01$).

Fig. 2). There was no association with the other patient and tumor characteristics in both breast and lung tumors.

DISCUSSION

The *APC* gene has been convincingly linked to the development of colorectal cancer. Whereas mutations within the mutation cluster region of the *APC* gene are rare in lung and breast cancers (7), functional somatic mutations outside the cluster region have been described in 18% of breast cancers (5). High rates of allelic loss at chromosome locus *5q21* and other findings suggest that APC inactivation may also play a role in lung and breast cancer pathogenesis. Moreover, female mice carrying a germ-line *APC* mutation develop both intestinal and mammary tumors (24). Decreased expression of APC and up-regulation or cellular relocation of β-catenin have been described in human lung and breast cancers and cell lines and in their animal models (25–29). *APC* and *β-catenin* mutations appear during the multistage development of carcinogen-induced rat lung tumors (30). Occasional somatic mutations of the *PP2A* gene (31), whose protein product interacts with β-catenin (32), are additional evidence of disruption of the Wnt signaling pathway in lung cancers.

Aberrant methylation of CpG-rich sites in gene promoter regions is recognized as an alternate mechanism to gene mutations for the transcriptional silencing of many TSGs (33). We and others have demonstrated that the promoter regions of several genes are aberrantly methylated in lung and breast cancers (20, 34–39). In this report we demonstrate that promoter 1A of APC was hypermethylated in 44% of breast cancer tumors and cell lines, in 53% of NSCLC tumors and cell lines, and in 26% of SCLC cell lines. One of us (J. H.) has previously reported a low frequency of APC promoter methylation in NSCLC and breast cancers (13). For this reason, the Gazdar and Herman laboratories exchanged samples of breast and lung cancer cell line DNAs and analyzed them in a blinded manner. Both laboratories obtained similar frequencies of methylation and the concordance between their results was 87%. The reasons for the previous low positive frequency from the Herman laboratory are not known, but may reflect the smaller number of samples tested. In colorectal carcinomas, the *APC* gene promoter 1A is methylated in about 18% of sporadic tumors but not in adjacent nonmalignant mucosa (11, 13). In gastric cancer, selective methylation of promoter 1A and silencing of its transcript (but not of promoter 1B) is frequently present both in cancerous tissue and in adjacent nonmalignant mucosa (12). Our findings indicate that only occasional methylation was present in nonmalignant samples adjacent to tumor, and it was absent in other normal tissues from healthy subjects.

Our results indicate that most cell lines tested contained either the unmethylated or the methylated form of promoter 1A, and only occasional cell lines contained both forms. By contrast, the unmethylated form was always present in tumors, presumably reflecting the presence of nonmalignant cells. Tumor cell lines (which represent pure populations of malignant cells) having both methylated and unmethylated forms lacked expression. Whether this reflects heterogeneity of methylation or whether the unmethylated allele is silenced by another mechanism is not known. There was complete concordance between promoter 1A methylation and the loss of its transcript. Demethylation Aza-CdR treatment restored transcript 1A, and there was expression in all eight-methylated cell lines. LOH at the *APC* locus (*5q21–22*) was observed in a high proportion of SCLC, NSCLC, and breast cancer cell lines. A high frequency of LOH at *5q21–22* was present in most of the methylated (10 of 15; 67%) cell lines, suggesting a mechanism for biallelic inactivation. However a high frequency of LOH was also found in unmethylated cell lines. Thus, although APC remains a likely target, the allelic loss in this region could be targeting other genes. The frequency of methylation in breast cancers increased with tumor stage and size, suggesting that methylation may be associated with poor prognosis. Our results and those of others (40) indicate that the *APC* gene product and the 1A transcript are expressed in normal bronchial and breast epithelial cells.

In lung and breast cancers, as with colorectal (13) and gastric cancers (12), methylation and the lack of expression of APC are limited to the 1A promoter and its transcript. The consequences of *APC* gene promoter 1A methylation and the loss of expression of its specific transcript are not entirely clear. Breast and lung tumors frequently have weaker *APC* gene immunostaining than their adjacent nonmalignant epithelial

cells.[4] Loss of expression was observed in tumors irrespective of APC promoter 1A methylation. Similar results have been described for BRCA1 promoter methylation in the majority of sporadic breast and ovarian tumors that had unmethylated, nonmutant *BRCA1* genes (41), suggesting that additional mechanisms may be responsible for reduced expression of these genes in the various tumors studied. Methylation of a single promoter has been described for other genes having multiple promoters, including *RARβ* (20) and *RASSF1* (35, 42). In these genes, as with *APC*, methylation and loss of transcript expression are highly selective and always involve only a single specific promoter—the other promoter is never methylated. Esteller et *al.* (13) correlated the methylation status of the 1A promoter with mutations of the *APC* gene in 66 colorectal cancers. Aberrant methylation was present in 5 of 19 (26%) cases with wild-type *APC*, but only in 3 of 47 (6%) tumors with a mutant gene ($P = 0.04$). Thus methylation was biased toward tumors with a genetically intact gene. We noted a significant trend between tumor size or stage and methylation frequency in breast cancers. Both tumor size and stage are negative prognostic factors for breast cancer, suggesting that aberrant methylation of the APC promoter 1A is associated with breast cancer progression.

In summary, aberrant methylation of the 1A promoter of the *APC* gene and loss of its specific transcript is frequent in breast and NSCLC cancers and cell lines and, to a lesser extent, in SCLC cell lines. Strong circumstantial evidence indicates that these findings may be of biological and clinical importance.

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Loss of Expression and Aberrant Methylation of the *CDH13* (H-Cadherin) Gene in Breast and Lung Carcinomas[1]

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ABSTRACT

Expression of some members of the cadherin family is reduced in several human tumors, and CDH13 (H-cadherin), located on chromosome 16q24.2–3, may function as a tumor suppressor gene. In human tumors, loss of expression of many tumor suppressor genes occurs by aberrant promoter region methylation. We examined the methylation status of the *CDH13* promoter in breast and lung cancers and correlated it with mRNA expression using methylation-specific PCR and reverse transcription-PCR. Methylation was frequent in primary breast tumors (18 of 55, 33%) and cell lines (7 of 20, 35%). In lung cancers, methylation was present more frequently in non-small cell lung cancer tumors (18 of 42, 43%) and cell lines (15 of 30, 50%) than in small cell lung cancer cell lines (6 of 30, 20%; $P = 0.03$). Only the methylated or unmethylated forms of the gene were present in most (73 of 80, 91%) tumor cell lines. *CDH13* expression was present in 24 of 30 (80%) of nonmethylated tumor lines. All 18 methylated lines tested lacked expression irrespective of whether the unmethylated form was present, confirming biallelic inactivation in methylated lines. Gene expression was restored in all five methylated cell lines tested after treatment with the demethylating agent 5′-aza-2-deoxycytidine. Our results demonstrate frequent aberrant methylation of *CDH13* in breast and lung cancers accompanied by loss of gene expression, although expression may occasionally be lost by other mechanisms.

INTRODUCTION

The cadherins are a family of cell surface glycoproteins responsible for selective cell recognition and adhesion (1). Several family members, including *CDH1* (E-cadherin) and *CDH13* (H-cadherin) are located on the long arm of chromosome 16 (16q) (2), while another gene cluster resides on the short arm of chromosome 5 (3). The chromosomal locations of several of the cadherins are sites of frequent LOH[3] in many tumor types (2). Deletions of 16q are frequent in breast, lung, and other carcinomas (4–8). Loss of expression of cadherins has been described in many epithelial cancers, and it may play a role in tumor cell invasion and metastasis (1, 9–11).

Although allelic loss is frequent in these cancers, Knudson's hypothesis (12) states that both alleles of a tumor suppressor gene have to be inactivated for tumorigenesis. Inactivation of the second allele may occur via point mutations, homozygous deletions, or by aberrant methylation. Aberrant methylation of 5′ gene promoter regions associated with gene silencing is an epigenetic phenomenon observed in many cancer types (13), and the number of methylated genes in individual cancers is estimated to be very high (14). CDH1 is the prototype cadherin family member, and its role in tumorigenesis has been studied extensively (11). *CDH1* may be inactivated by multiple mechanisms (15). In breast, prostate, and thyroid cancers, 5′ CpG island promoter methylation is a frequent method of *CDH1* inactivation (16, 17). However, the methylation patterns of the *CDH1* in breast cancers are unstable and reflect a dynamic, heterogeneous loss of gene expression during metastatic progression (18).

CDH13 expression is diminished in breast and lung cancers (7, 19). In ovarian tumors, the combination of deletion and aberrant methylation has been reported to inactivate *CDH13* (20). Aberrant methylation of *CDH13* has also been reported in lung cancers (7). The purpose of this study was to determine the frequency of *CDH13* methylation in human breast and lung cancers and to investigate the relationship between aberrant methylation and expression of *CDH13* in tumor cell lines.

MATERIALS AND METHODS

Clinical Samples. Surgically resected specimens from 55 primary breast tumors and 17 corresponding nonmalignant breast tissues from these patients were obtained from the Tumor and Tissue Repository at the Hamon Center. Tumor samples from 42 primary NSCLC (11 squamous cell and 31 adenocarcinomas) and 25 corresponding nonmalignant lung tissues were obtained from surgical resections performed at the M. D. Anderson Cancer Center. For gene expression studies, six nonmalignant tissue samples (two breast, two peripheral lung tissues, and one bronchial mucosa) were obtained as far from the tumor tissue as possible. Epithelial cells from buccal swabs of 8 healthy nonsmoking volunteers and peripheral blood lymphocytes from 12 healthy volunteers also were obtained. Appropriate Institutional Review Board permission was obtained from both participating centers, and written informed consent was obtained from all subjects. Tissues were stored at −80°C for up to 3 years prior to testing.

Cell Lines. Human tumor cell lines (20 breast lines, 30 SCLC lines, and 30 NSCLC lines) and corresponding B-lymphoblastoid lines ($n = 47$) were established by us (21, 22). Most breast and NSCLC lines were established from primary tumors, and most SCLC lines were established from metastases. Cells cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO_2 at 37°C.

DNA Extraction. Genomic DNA was obtained from cell lines, primary tumors, and nonmalignant cells by digestion with proteinase K (Life Technologies, Inc.) for 1 day at 37°C, followed by two extractions with phenol: chloroform (1:1) (23).

MSP. Aberrant promoter methylation of *CDH13* was determined by the method of MSP as reported by Herman *et al.* (24) using primers specific for *CDH13*-methylated and -unmethylated sequences (7). Forward and reverse primers for the methylated sequence were 5′-TCGCGGGGTTCGTTTT-TCGC-3′ and 5′-GACGTTTTCATTCATACACGCG-3′, respectively, and for the unmethylated sequence were 5′-TTGTGGGGTTGTTTTTTGT-3′ and 5′-AACTTTTCATTCATACACACA-3′, respectively. Briefly, 1 μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all unmethylated cytosines to uracil while methylated cytosines remain unchanged (25). The modified DNA was purified using a Wizard DNA purification kit (Promega, Madison, WI), treated with NaOH to desulfonate, precipitated with ethanol, and resuspended in water. Two sets of primers were

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[3] The abbreviations used are: LOH, loss of heterozygosity; MSP, methylation-specific PCR; SCLC, small cell lung cancer; NSCLC, non- small cell lung cancer; RT, reverse transcription; Aza-CdR, 5-aza-2′-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 *Incidence of methylation in breast and lung tumors, cell lines, and nonmalignant control tissues*

Samples	Total no.	No. methylated (%)
Breast		
Cell lines	20	7 (35)
Primary tumors	55	18 (33)
Total breast cancer samples	75	25 (33)
Lung		
SCLC		
Cell lines	30	6 (20)[a]
NSCLC		
Cell lines	30	15 (50)[a]
Primary tumors	42	18 (43)
Total NSCLC samples	72	33 (46)
Nonmalignant		
Peripheral blood lymphocytes[b]	12	0 (0)
Epithelial cells from buccal swabs[b]	8	0 (0)
Nonmalignant breast[c]	17	1 (6)
Nonmalignant peripheral lung[c]	25	2 (8)
Total nonmalignant samples	62	3 (5)

[a] The differences in methylation incidences between SCLC and NSCLC lines were significant ($P = 0.03$) for cell lines.
[b] From healthy volunteers.
[c] From resections for breast and lung cancers.

used to amplify each region of interest: PCR amplification was done with bisulfite-treated DNA as template using specific primer sequences for the methylated (*i.e.*, unmodified by bisulfite treatment) and unmethylated (*i.e.*, bisulfite modified to UpG) forms of the gene. DNA from peripheral blood lymphocytes ($n = 12$) and buccal mucosae ($n = 8$) from healthy nonsmoking subjects were used as negative controls for methylation-specific assays. DNA from lymphocytes of healthy volunteers treated with Sss1 methyltransferase (New England Biolabs, Beverly, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. Water blanks were included with each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples.

Expression of *CDH13*. Expression of *CDH13* was analyzed by the RT-PCR technique. Total RNA was extracted from the cell lines (20 breast, 19 NSCLC, and 9 SCLC cell lines) with TRIzol (Life Technologies, Inc.) following the manufacturer's instructions. RT reaction was performed on 2 μg of total RNA with the SuperScript II First-Strand Synthesis using an oligo(dT) primer system (Life Technologies, Inc.). Primer sequences and conditions for RT-PCR product were previously described (forward primer, 5′-TTCAGCAGAAAGTGTTCCATAT-3′ in exon 2 and reverse primer, 5′-GTGCATGGACGAACAGAGT-3′ in exon 3) (7) and confirmed that genomic DNA was not amplified with these primers. The housekeeping gene *GAPDH* was used as an internal control to confirm the success of the RT reaction (forward primer, 5′-ACAGTCCATGCCATCACTGCC-3′ and reverse primer, 5′-GCCTGCTTCACCACCTTCTTG-3′) (26). PCR products were analyzed on 2% agarose gels.

Aza-CdR Treatment. Five tumor cell lines with *CDH13* promoter methylation and absent gene expression were incubated in culture medium with and without Aza-CdR at a concentration of 2 μg/ml for 6 days, with medium changes on days 1, 3, and 5 (27). Cells were harvested at the end of the sixth day for extraction of total RNA and tested for gene expression.

Analysis of LOH. Three polymorphic microsatellite markers (*D16S422*, *D16S3098*, and *D16S511*) located at chromosome region 16q24.2–3 were selected for LOH analysis. DNA from 13 breast cancers, 9 SCLC, and 14 NSCLC cell lines and their corresponding B-lymphoblastoid lines (as sources of constitutional DNA) were analyzed (28). Briefly, 100 ng of genomic DNA was amplified by PCR in the presence of [^{32}P]CTP using the microsatellite marker. PCR products were separated by electrophoresis in 6% polyacrylamide gels containing 7 M urea and were visualized by autoradiography. Subjects who yielded two distinguishable bands of different sizes but similar intensity in the lane having normal DNA were termed informative (*i.e.*, heterozygous) for the marker. Samples having only a single major band in normal DNA were termed noninformative. LOH was defined as complete loss of a band corresponding to an allele present in informative cases.

DNA Sequencing. The MSP products of five cell lines with promoter methylation were isolated from the gels and purified. After amplification with the same primers used for MSP, 20 ng of PCR products were sequenced by the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA). In addition, we amplified 411 nucleotides encompassing nucleotides 1315 and 1725 of the 5′ region of the *CDH13* gene (accession no. AB001090) in bisulfite treated genomic DNA by primers we designed (forward, 5′-TTGGAAAAGTGGAATTAGTTGG-3′; reverse, 5′-CCTCTTCCCTACCTAAAACA-3′). These primers were designed to exclude binding to any CpG site and to ensure amplification of both methylated and unmethylated sequences. PCR products were sequenced from both ends after purification. This region included the MSP primer sites and amplicon and encompassed 24 CpG sites.

Data Analysis. Statistical differences between groups were examined using χ^2 tests and Fisher's exact test with continuity correction. Probability values of <0.05 were regarded as statistically significant.

RESULTS

Aberrant Promoter Methylation and Expression of *CDH13*. Results of aberrant promoter methylation of *CDH13* are detailed in Table 1 and representative examples are illustrated in Fig. 1*A*. Aberrant methylation was found in primary breast tumors (18 of 55, 33%)

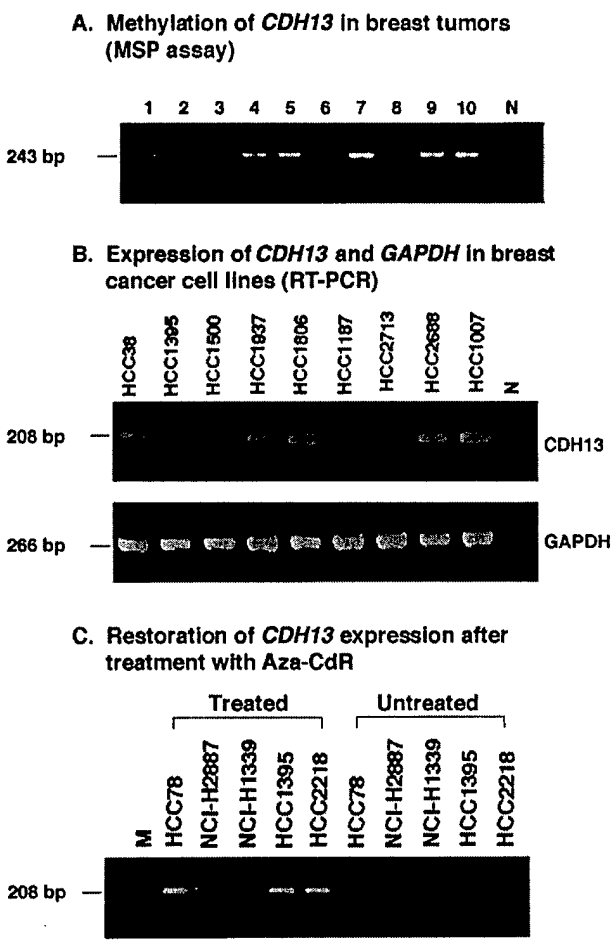

Fig. 1. Methylation and expression of *CDH13* in tumors and cell lines. *A*, presence of a methylated band (demonstrated by MSP assay) in 6 of 10 breast cancer specimens. *N*, blank control. *B*, *CDH13* expression (by (RT-PCR) in 7 of 10 breast cancer cell lines. *N*, blank control. Expression of the housekeeping gene *GAPDH* was run as a control for RNA integrity. *C*, lack of *CDH13* expression in five methylated cell lines in the untreated state and restoration of gene expression after Aza-CdR treatment. The cell lines were derived from NSCLC (HCC78 and NCI-H2887, SCLC (NCI-H1339) or breast (HCC1395 and HCC2218) cancers.

Table 2 *Methylation status of CDH13 in breast and lung cancer cell lines*

Methylation status		No. with phenotype (n = 80)
Unmethylated band	Methylated band	
Absent	Absent	0
Present	Absent	52 (65%)
Absent	Present	21 (26%)
Present	Present	7 (9%)

and cell lines (7 of 20, 35%). In lung cancers, aberrant methylation was present more frequently in NSCLC primary tumors (18 of 42, 43%; including 3 of 11 squamous cell carcinomas and 15 of 31 adenocarcinomas) and cell lines (15 of 30, 50%) than in SCLC cell lines (6 of 30, 20%; $P = 0.03$ between cell lines; Table 1). Differences in frequencies between breast and NSCLC tumors and their respective cell lines were not significant. Only the methylated or unmethylated forms of the gene were present in 73 of 80 (91%) cell lines and both forms were present in 7 of 80 (9%) cell lines (Table 2). Aberrant methylation was absent in DNA from peripheral blood lymphocytes and buccal swabs from volunteers. In DNA from nonmalignant tissues from breast and NSCLC resections, aberrant methylation was present in 1 of 17 breast and 2 of 25 lung nonmalignant samples (Table 1). The corresponding tumor samples were also methylated in the three cases where the nonmalignant tissues were methylated. In tumor samples, most of which consist of mixtures of tumor cells and nonmalignant cells, either the unmethylated band only or both methylated and unmethylated bands were present (data not shown). The presence of unmethylated *CDH13* promoter sequences in all of the tissues analyzed confirmed the integrity of the DNA in these samples.

RT-PCR revealed *CDH13* expression in control tissues (samples of nonmalignant breast, peripheral lung, and bronchial mucosa). However, loss of *CDH13* expression was present in 10 of 20 (50%) breast cancers, 3 of 9 (33%) SCLC, and 11 of 19 (58%) NSCLC cell lines (Table 3A). Sample gels are shown in Fig. 1*B*. Expression of *GAPDH* was used as the internal control (Fig. 1*B*). All 18 tumor lines with *CDH13* methylation lacked gene expression irrespective of whether the unmethylated form was present. Expression was present in 24 of 30 nonmethylated lines. Gene expression was restored in five methylated cell lines (two breast, one SCLC, and two NSCLC) after treatment with the demethylating agent Aza-CdR (Fig. 1*C*).

LOH in Breast and Lung Cancer Cell Lines. We determined LOH in a panel of 13 breast and 23 lung cancer cell lines (9 SCLC and 14 NSCLC) that were paired with corresponding B-lymphoblastoid cell lines using three polymorphic markers (*D16S422*, *D16S3098*, and *D16S511*; Fig. 2). Although the markers are located at the same chromosomal band location as *CDH13*, the exact relationship of the markers to the gene is not known. We found LOH for one or more markers in 8 of 13 (62%) breast, in 4 of 9 (44%) SCLC, and in 5 of 14 (36%) NSCLC cell lines (Table 3B). LOH at one or more markers was present in five of six (83%) breast cancers, in two of two (100%) SCLC, and in four of seven (57%) NSCLC cell lines in which the *CDH13* promoter was methylated (Table 3B). The overall concordance between promoter methylation and LOH was 72%.

DNA Sequencing of MSP Products. We sequenced MSP products of methylated *CDH13* in five cell lines (breast cancer lines HCC2157 and HCC2218 and lung cancer lines NCI-H2087, NCI-H2196, and NCI-H2887). There were seven CpG sites in the amplicon excluding the primer sites. All of the CpG sites were methylated in all samples except one site in the NCI-H2218 cell line.

Using methylation-independent primers, we amplified and sequenced the 5′ region of the *CDH13* gene which included the MSP primer attachment sites and their amplicon. Two cell lines (NSCLC line NCI-H1770 and breast cancer line HCC38) lacking methylated

Table 3 *Relationship between expression, methylation of CDH13, and LOH in chromosome 16q24.2-3 in breast and lung cancer cell lines*[a]

A. Data for individual breast and cancer cell lines

Tumor cell line	Expression (RT-PCR)	Methylation status (MSP)	LOH
Breast			
HCC38	Present	Unmethylated	Absent
HCC1395	Absent	Methylated	Present
HCC1500	Absent	Both	Not done
HCC1937	Present	Unmethylated	Present
HCC1187	Present	Unmethylated	Present
HCC2713	Absent	Methylated	Present
HCC2688	Present	Unmethylated	Not done
HCC70	Present	Unmethylated	Not done
HCC202	Present	Unmethylated	Not done
HCC712	Present	Unmethylated	Absent
HCC1007	Present	Unmethylated	Absent
HCC1419	Present	Unmethylated	Not done
HCC1428	Absent	Unmethylated	Absent
HCC1569	Absent	Unmethylated	Not done
HCC1739	Absent	Unmethylated	Present
HCC1806	Present	Unmethylated	Not done
HCC1954	Absent	Methylated	Absent
HCC2157	Absent	Methylated	Present
HCC2185	Absent	Methylated	Present
HCC2218	Absent	Methylated	Present
SCLC			
HCC33	Present	Unmethylated	Absent
NCI-H209	Present	Unmethylated	Present
NCI-H1339	Absent	Methylated	Present
NCI-H1450	Present	Unmethylated	Absent
NCI-H1607	Present	Unmethylated	Absent
NCI-H1963	Present	Unmethylated	Absent
NCI-H2107	Present	Unmethylated	Absent
NCI-H2141	Absent	Methylated	Present
NCI-H2195	Absent	Unmethylated	Present
NSCLC			
HCC15	Absent	Methylated	Absent
HCC78	Absent	Methylated	Absent
HCC44	Present	Unmethylated	Absent
HCC95	Present	Unmethylated	Not done
HCC366	Absent	Methylated	Present
HCC515	Absent	Methylated	Present
HCC193	Present	Unmethylated	Absent
HCC827	Absent	Unmethylated	Not done
HCC1171	Absent	Unmethylated	Absent
NCI-H1395	Absent	Both	Absent
HCC1437	Present	Unmethylated	Absent
NCI-H1819	Present	Unmethylated	Present
NCI-H1770	Present	Unmethylated	Absent
NCI-H1993	Present	Unmethylated	Not done
NCI-H2009	Absent	Methylated	Present
NCI-H2087	Absent	Methylated	Present
NCI-H2122	Absent	Both	Not done
NCI-H2126	Present	Unmethylated	Absent
NCI-H2887	Absent	Methylated	Not done

B. Summaries of the different phenotypes identified

Expression (RT-PCR)	LOH	Methylation status	Cancer cell lines (n = 36) Breast (n = 13)	SCLC (n = 9)	NSCLC (n = 14)	Total no.
Present	Present	Unmethylated	2	1	1	
		Methylated	0	0	0	4
		Both	0	0	0	
	Absent	Unmethylated	3	5	5	
		Methylated	0	0	0	13
		Both	0	0	0	
Absent	Present	Unmethylated	1	1	0	
		Methylated	5	2	4	13
		Both	0	0	0	
	Absent	Unmethylated	1	0	1	
		Methylated	1	0	2	6
		Both	0	0	1	

[a] For methylation status, the results of MSP assays using primers specific for the methylated or unmethylated forms are presented. Note for three cells lines (breast cell line HCC1500 and NSCLC lines NCI-H1395 and NCI-H2122) both methylated and unmethylated bands were present.

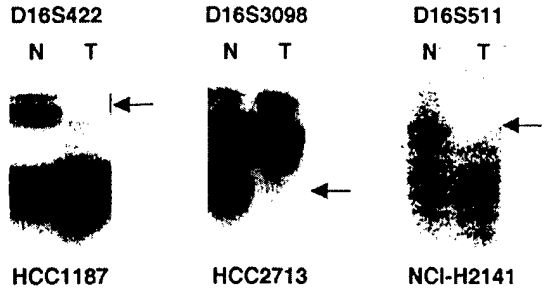

Fig. 2. Representative examples of autoradiographs demonstrating LOH in breast and lung cancer cell lines and using three microsatellite makers for chromosome 16q24.2–3 (*D16S422*, *D16S3098*, and *D16S511*). *N*, normal sample (corresponding B-lymphoblastoid line); *T*, tumor cell line. Examples of breast (HCC1187 and HCC2713) and SCLC (NCI-H2141) cell lines are illustrated.

bands after MSP assay were completely unmethylated at all 24 CpG sites in the methylation-independent amplicon. Of the three lines that yielded a product after MSP assay (NSCLC cell lines NCI-2087 and NCI-2887 and breast cancer line HCC2157), all nine CpG sites at forward (five sites) and reverse (four sites) primer attachments were fully methylated. However, the seven CpG sites that were encompassed in the MSP amplicon region showed heterogeneity of methylation.

DISCUSSION

Decreased expression of cadherin molecules in invasive carcinomas results in cell scattering and decreased mediated cell-cell adhesion (29–31), which may enhance tumor progression and invasion. Although the role of CDH1 has been studied extensively, there is evidence that CDH13 also may function as a tumor suppressor gene. *CDH13* is located at chromosome 16q24.2–3, a site of frequent LOH in several human cancers (5, 32, 33), and expression of *CHD13* is significantly reduced in some breast, lung, and ovarian cancers (7, 19, 20, 34).

A frequent method of suppression of tumor suppressor gene function is via aberrant methylation of the promoter region resulting in down-regulation of gene expression. In lung cancers, aberrant promoter methylation *CDH13* has been reported without mutation (7). However, it is important to demonstrate the biological relevance of gene methylation (28, 35, 36). We and others have described the criteria required for the demonstration of biological significance (35, 37): (*a*) aberrant methylation is frequent in the tumor type studied; (*b*) methylation is a rare event in nonmalignant and control tissues; (*c*) loss of expression is a frequent event in tumors; (*d*) aberrant methylation and gene expression are correlated with each other; (*e*) gene expression is restored after exposure to a demethylating agent; and (*f*) there is a high frequency of 16q allelic loss in the tumors, suggesting a mechanism for biallelic loss. However, because the polymorphic regions examined for allelic loss are perigenic rather than intragenic, the loss may have targeted other genes in the region including *CDH1* and certain other members of the *CDH* family.

With the assay conditions used, all control tissues from healthy volunteers were negative for *CDH13* promoter methylation, and the gene was expressed in nonmalignant breast tissues, peripheral lung, and bronchial epithelium. Only occasional methylation was present in nonmalignant tissues adjacent to cancers, whereas a relatively high percentage of breast and NSCLC tumors and cell lines (33–50%) were positive. While heterogeneity of methylation was noted in the 5′ region of the gene, the MSP primer attachment sites were consistently methylated in three MSP-positive cell lines and completely unmethylated in two MSP-negative cell lines. Thus, the MSP results would be expected to be consistent even if the resultant amplicon showed heterogeneity of methylation. The excellent concordance (88%) between the positive MSP assay result and loss of *CDH13* expression provides powerful evidence that the MSP assay results correlate with gene silencing. Treatment with Aza-CdR restored transcript expression in methylated cell lines, confirming that methylation was responsible for loss of gene expression in these lines.

The methylation frequencies in both breast and NSCLC tumors were not significantly different from their respective cell lines, indicating that cell lines are suitable models for studying *CDH13* promoter methylation. Although SCLC tumors were not available for study, the frequency of methylation in SCLC cell lines (20%) was significantly lower than the frequency in NSCLC cell lines (50%). Significant differences between SCLC and NSCLC have been reported for the methylation frequencies of other genes (38, 39), suggesting that the two major forms of lung cancer arise via different pathogenetic pathways.

Knudson's hypothesis (12) states that both alleles of a tumor suppressor gene have to be inactivated for tumorigenesis. In ovarian tumors, inactivation of *CDH13* has been reported to occur by the combination of allelic loss and aberrant methylation (20). Although there was a relatively high frequency of allelic loss in breast and lung cell lines, the concordance between LOH at chromosome 16q24.2–3 and methylation status was moderate (72%). However, most methylated cell lines lacked an unmethylated allele, indicating biallelic inactivation. Because 20% of unmethylated cell lines lacked gene expression, alternative methods of gene silencing must exist in some tumors. Of interest, binding of the transcription factor Snail to the *CDH1* promoter results in gene silencing (40, 41). It is not known whether Snail binds to *CDH13*.

Our results strongly suggest that silencing of *CDH13* gene expression by methylation plays a role in the pathogenesis of breast and lung cancers. Future studies, including transfection of the gene, will be required to identify the biological effects of gene silencing and their relationship to the malignant phenotype.

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Epigenetic Inactivation of RASSF1A in Lung and Breast Cancers and Malignant Phenotype Suppression

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Background: **The recently identified RASSF1 locus is located within a 120-kilobase region of chromosome 3p21.3 that frequently undergoes allele loss in lung and breast cancers. We explored the hypothesis that RASSF1 encodes a tumor suppressor gene for lung and breast cancers.** ***Methods:*** **We assessed expression of two RASSF1 gene products, RASSF1A and RASSF1C, and the methylation status of their respective promoters in 27 non-small-cell lung cancer (NSCLC) cell lines, in 107 resected NSCLCs, in 47 small-cell lung cancer (SCLC) cell lines, in 22 breast cancer cell lines, in 39 resected breast cancers, in 104 nonmalignant lung samples, and in three breast and lung epithelial cultures. We also transfected a lung cancer cell line that lacks RASSF1A expression with vectors containing RASSF1A complementary DNA to determine whether exogenous expression of RASSF1A would affect *in vitro* growth and *in vivo* tumorigenicity of this cell line. All statistical tests were two-sided.** ***Results:*** **RASSF1A messenger RNA was expressed in nonmalignant epithelial cultures but not in 100% of the SCLC, in 65% of the NSCLC, or in 60% of the breast cancer lines. By contrast, RASSF1C was expressed in all nonmalignant cell cultures and in nearly all cancer cell lines. RASSF1A promoter hypermethylation was detected in 100% of SCLC, in 63% of NSCLC, in 64% of breast cancer lines, in 30% of primary NSCLCs, and in 49% of primary breast tumors but in none of the nonmalignant lung tissues. RASSF1A promoter hypermethylation in resected NSCLCs was associated with impaired patient survival ($P = .046$). Exogenous expression of RASSF1A in a cell line lacking expression decreased *in vitro* colony formation and *in vivo* tumorigenicity.** ***Conclusion:*** **RASSF1A is a potential tumor suppressor gene that undergoes epigenetic inactivation in lung and breast cancers through hypermethylation of its promoter region. [J Natl Cancer Inst 2001;93:691–9]**

Allelic loss of human chromosome 3p is an early and frequent event in the development of several cancers, including lung and breast cancers *(1–5)*. Identification of a series of nested 3p21.3 homozygous deletions in small-cell lung cancers (SCLCs) directed an intensive effort to positionally clone tumor suppressor genes from a 630-kilobase (kb) region, which was recently narrowed to a 120-kb subregion by identification of a breast cancer homozygous deletion *(6–8)*. Sequencing the entire 630-kb region identified at least 25 genes, several of which may encode tumor suppressor genes for lung cancer *(7)*. Nine genes are located in or on the border of the breast cancer-defined subregion. One of these genes, which spans 7.6 kb of genomic DNA, has a predicted Ras-association domain and homology to the Ras-effector Norel (Fig. 1); it has, therefore, been termed "RASSF1" *(9,10)*.

The RASSF1 gene encodes two major transcripts, RASSF1A and RASSF1C, which are produced by alternative promoter selection and alternative messenger RNA (mRNA) splicing. RASSF1A is encoded by RASSF1 exons 1A, 1C, and 2–5. RASSF1C is encoded by RASSF1 exons 1–5 (Fig. 1). The start sites for RASSF1A and RASSF1C are approximately 2 kb apart and have two independent CpG island-containing putative promoter regions. RASSF1A is predicted to encode a 39-kd peptide that contains an N-terminal diacylglycerol (DAG)-binding domain and a Ras-association domain (Fig. 1). RASSF1C is predicted to encode a 32-kd peptide that lacks a DAG-binding domain but contains a Ras-association domain *(7,11)*. Immediately adjacent to the DAG-binding domain of RASSF1A is a sequence PxxP, which is the minimal sequence required for an src homology 3-binding domain. RASSF1A has a central linker that contains a number of prolines, as well as acidic and hydroxyl-bearing residues. These regions, called PEST sequences, are found in proteins that are rapidly turned over by ubiquitination-dependent pathways *(12)*. For RASSF1C, the amino-terminal region unique to this isoform is enriched for these PEST sequences. Within the PEST sequences common to both RASSF1A and RASSF1C is a serine residue that is phosphorylated *in vitro* by DNA-dependent ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia-related kinases *(13)*. The Ras-association domain is more than 50% identical and more than 70% similar to the carboxyl terminal 225 residues of mouse Norel *(10)*. The Ras-association domain, consisting of a core of 90 amino acids, is flanked on the amino terminal side by a region homologous with a region found in Norel and *Caenorhabditis elegans* orthologue T24F1.3 protein.

In this article, we characterized RASSF1A and RASSF1C as potential tumor suppressor genes in lung and breast cancers.

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See "Notes" following "References."

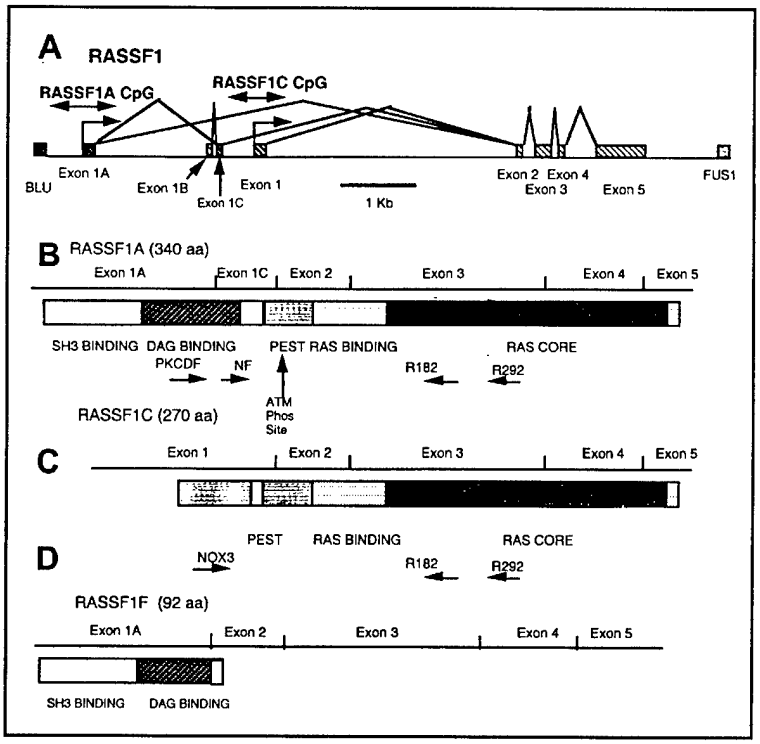

Fig. 1. Map of the RASSF1 locus, transcripts, and protein domains. **A)** The exon–intron structure of the RASSF1 locus with the location of the CpG islands in the predicted promoter regions (the locations of which are shown by **double-headed arrows**) of RASSF1A and RASSF1C. RASSF1A transcription is predicted to come from the most centromeric promoter region located within a CpG island and begins with exon 1A. RASSF1F also commences at this promoter but is missing exon 1C. Transcription of RASSF1C is predicted to begin in the most telomeric promoter region, which is approximately 2 kilobases from that of RASSF1A and begins with exon 1. **Blocks** represent exons; **lines** represent introns. **B)** Schematic of the RASSF1A transcript and predicted protein-sequence domains. The location of the various primers (PKCDF, NF, R182, and R292) used for isoform-specific reverse transcription (RT)–polymerase chain reaction (PCR) analyses are indicated. **Tick marks** identify the exon boundaries. The potential src homology 3 (SH3)-binding region, putative diacylglycerol (DAG)-binding domain, PEST sequence, Ras-association domain, and ataxia-telangiectasia-mutated (ATM) phosphorylation site are labeled. **C)** Schematic of the RASSF1C transcript and predicted protein-sequence domains. The locations of the various primers (NOX3, R182, and R292) used for isoform-specific RT–PCR analyses are indicated. **D)** Schematic of the RASSF1F transcript and predicted protein-sequence domains.

Because loss of gene expression can be caused by tumor-acquired aberrant methylation, we assessed the methylation status of the RASSF1A promoter region in these tumors *(14)*. In addition, we tested the ability of RASSF1A to suppress the malignant phenotype. Previously, Dammann et al. *(15)* showed that the RASSF1A promoter is hypermethylated in lung cancer cells and that exogenous expression of RASSF1A suppresses tumorigenesis in nude mice. We have confirmed and extended those findings by analyzing the expression and methylation status of both the RASSF1A and RASSF1C genes in lung and breast cancers.

Methods

Patient Population

Resected lung tumor samples and clinical data were collected from patients after obtaining appropriate institutional review board approval and patients' written informed consent. Primary tumor samples and corresponding noninvolved lung tissues were obtained from 107 patients with non-small-cell lung carcinoma (NSCLC) who had received curative resection surgery at the Prince Charles Hospital, Brisbane, Australia, from June 1990 through March 1993, and for whom clinical and survival data of 5 or more years were available *(16,17)*. Among the 107 patients, there were 76 males and 31 females (range, 28–81 years; mean age, 61 years at diagnosis). Among the patients, 61 had stage I cancers, 21 had stage II, 24 had stage IIIA, and one had stage IIIB *(18)*. Histologically, there were 45 adenocarcinomas, 43 squamous cell carcinomas, 11 adenosquamous carcinomas, four large-cell carcinomas, three atypical carcinoids, and one typical carcinoid. Ninety-eight patients were smokers, with a mean exposure of 31 pack-years, and nine were never smokers or nonsmokers.

We also obtained 39 primary breast tumors from patients aged 31–84 years undergoing breast cancer treatment in The University of Texas Southwestern Hospital system. Among the patients, three had stage I cancers, 15 had stage IIA, two had stage IIB, eight had stage IIIA, five had stage IIB, and six had stage IV. Histologically, there were 30 infiltrating ductal carcinomas, four invasive lobular carcinomas, one lobular carcinoma *in situ*, two ductal carcinomas *in situ*, and two breast adenocarcinomas at metastatic sites. Clinical information was obtained by retrospective review of clinical records.

Cell Lines and Cell Cultures

Lung and breast tumor cell lines generated by us have been described previously *(19–21)*. Complementary DNAs (cDNAs) and genomic DNAs were obtained from cell lines, most of which have been deposited in the American Type Culture Collection (ATTC) (Manassas, VA), that represented the spectrum of lung cancer histologies. These cell lines include the following: (all Hxxxx lines have the prefix National Cancer Institute [NCI]-) SCLCs (i.e., H69, H82, H128, H146, H182, H187, H196, H209, H249, H289, H290, H345, H378, H524, H526, H592, H735, H738, H740, H748, H774, H841, H847, H862, H889, H1092, H1105, H1184, H1304, H1339, H1450, H1607, H1618, H1672, H1688, H1963, H2028, H2029, H2081, H2108, H2171, H2195, H2227, and HCC970) and NSCLCs (i.e., H23, H28, H125, H157, H226, H358, H720, H727, H838, H920, H1155, H1299, H1437, H1466, H1573, H1648, H1770, H1792, H1819, H1838, H1993, H2009, H2052, H2077, H2087, H2347, H2452, H2882, H2887, HCC44, HCC78, HCC95, HCC193, HCC515, HCC827, and HCC1171). Breast cancer cell lines used in these studies were the following: HTB19, HTB20, HTB22, HTB23, HTB24, HTB25, HTB26, HTB27, HTB121, HTB130, HTB131, HTB132, HTB133 (all HTB lines are available from the ATCC), HCC38, HCC70, HCC202, HCC712, HCC1007, HCC1143, HCC1187, HCC1395, HCC1419, HCC1428, HCC1500, HCC1569, HCC1739, HCC1806, HCC1937, HCC1599, HCC1954, HCC2157, HCC2185, HCC2218, HCC2688, and HCC2713. Normal human bronchial epithelial (NHBE) and small-airway epithelial (SAE) cell cultures were obtained from Clonetics (San Diego, CA) and were grown and harvested as directed by the vendor.

Expression Analysis of RASSF1 Isoforms

The identification of the RASSF1 gene (initially called 123F2) and its major isoforms RASSF1C and RASSF1A was reported as part of the overall characterization of the genes in the larger 630-kb 3p21.3 homozygous-deletion region *(7)* (Fig. 1, A–C).

Sequence information from exons 1A and 3 was used to design the forward primer PKCDF (5′-GGCGTCGTGCGCAAAGGCC-3′) and the reverse primer R182 (5′-GGGTGGCTTCTTGCTGGAGGG-3′) (Fig. 1, C). This primer pair

was used in reverse transcription (RT)–polymerase chain reaction (PCR) screens of lung, heart, and pancreatic tissue-specific cDNA libraries (Clontech Laboratories, Inc., Palo Alto, CA). The conditions used TaqGold (The Perkin-Elmer Corp., Norwalk, CT) with 1× TaqGold buffer adjusted to 2 m*M* $MgCl_2$. All reactions used a 70 °C–60 °C touchdown, with 5% dimethyl sulfoxide for 35 rounds (denaturation for 30 seconds, annealling for 30 seconds, and extension for 60 seconds) of PCR. The RASSF1A cDNA sequence is identical to that of the RASSF1C cDNA from the second exon to the carboxyl terminus, but the two cDNAs have different 5′ exons (RASSF1A, GenBank Accession #AF102770: exons 1A and 1C; RASSF1C, GenBank Accession #AF040703: exon 1 [Fig. 1]). We also isolated tissue-specific isoforms from the heart (RASSF1D, GenBank Accession #AF102771) and the pancreas (RASSF1E, GenBank Assession #AF102772) cDNA libraries.

Primers derived from exon–intron junctions (sequences available online at the Journal website) were used for genomic DNA single-strand conformation polymorphism mutation analysis of the coding regions of RASSF1A on a panel of NSCLC, SCLC, and breast cancer cell line DNAs. DNA was prepared from tumors and cell lines by standard methods *(22)*, and aberrantly migrating fragments were sequenced as described previously *(16,23)*.

RNA Analysis

Isoform-specific RT–PCR assays were used for analysis of RASSF1A and RASSF1C expression. Primers for RASSF1C were Nox3 (5′-CTGCAGCCAAGAGGACTCGG-3′) and R182 and for RASSF1A were either PKCDF or NF (5′-TGCAAGTTCACCTGCCAC-3′) and R182 (Fig. 1, C). Total RNA was isolated from previously described lung and breast cancer cell lines grown in RPMI-1640 medium supplemented with 5% fetal bovine serum (complete medium) *(19–21)* by Trizol extraction (Life Technologies, Inc. [GIBCO BRL], Rockville, MD). Four micrograms of total RNA was reverse transcribed by use of GIBCO-BRL Superscript First Strand cDNA Kit. All cDNA preparations were tested for the ability to amplify a nontranscribed genomic sequence immediately upstream of the first exon of the RASSF1A transcript. Any cDNAs that produced a product from this sequence were discarded because they were contaminated with genomic DNA.

We also assessed the expression of RASSF1A after exposure to 5-aza-2′-deoxycytidine, a drug that inhibits DNA methylation. We exposed subconfluent cultures of the RASSF1A-nonexpressing NSCLC line NCI-H157 to 0.5 μ*M* 5-aza-2′-deoxycytidine for 48 hours, after which we isolated total RNA and performed RT–PCR for RASSF1A, RASSF1C, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT–PCR of GAPDH transcripts was performed with the use of forward primer GAPDH-C (5′-CATGACAACTTTGGTATCGTG-3′) and reverse primer GAPDH-D (5′-GTGTCGCTGTTGAAGTCAGA-3′). RT–PCR products were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Methylation Analysis

The methylation status of the presumed RASSF1A and RASSF1C promoter regions was determined by methylation-specific PCR. Genomic DNAs from lung cancer cell lines not expressing RASSF1A (NCI lines H1299, H1184, H1304, H841, H2108, and H128) or expressing RASSF1A (H1792 and H2009) were modified by sodium bisulfite treatment as described previously *(24,25)*. Bisulfite treatment converts cytosine bases to uracil bases but has no effect on methylcytosine bases. PCR amplification followed by sequencing of the PCR fragments identifies specific CpG dinucleotides in the promoter region that are modified by methylation *(24,26,27)*. PCR primers (sequences available online at the Journal website) were designed to amplify genomic sequences in the presumed promoter regions of RASSF1A (cosmid Luca12; GenBank Accession #AC002481 nucleotides 17 730–18 370) and RASSF1C (GenBank Accession #AC002481 nucleotides 21 022–21 152 and 21 194–21 332). The resulting PCR fragments were sequenced by automated fluorescence-based DNA sequencing to determine the methylation status.

The data on CpG methylation in RASSF1A-nonexpressing lung cancer cell lines (data available online at the Journal website) were used to design methylation-specific PCR *(24)* primers for the RASSF1A 5′ promoter region: The primers to detect the methylated form were 5′-GGGTTTTGCGAGAGCGCG-3′ (forward) and 5′-GCTAACAAACGCGAACCG-3′ (reverse), and the primers to detect the unmethylated form were 5′-GGTTTTGTGAGAGTGTGTTTAG-3′ (forward) and 5′-CACTAACAAACACAAACCAAAC-3′ (reverse). Each primer set generated a 169-base-pair (bp) product. Methylation-specific PCR cycling conditions consisted of one incubation of 15 minutes at 95 °C, followed by 40 cycles of a 30-second denaturation at 94 °C, 50 seconds at an annealing temperature (64 °C for methylation-specific and 59 °C for unmethylated-specific primers), a 30-second extension at 72 °C, and a final extension at 72 °C for 10 minutes. PCR products were separated in 2% agarose gels. Lymphocyte DNA, methylated *in vitro* by CpG (*Sss*I) methylase (New England Biolabs, Inc., Beverly, MA) following the manufacturer's directions, was used as a positive control. A water blank was used as a negative control.

Generation of Transfectants

RASSF1A cDNA was cloned into pcDNA3.1+ (Invitrogen Corp., Carlsbad, CA), resequenced to confirm that the cDNAs were in the correct orientation and reading frame, transcribed, and translated *in vitro* with commercial kits (Clonetech Laboratories, Inc.). The expression vector containing RASSF1A produced a 42-kd protein, and the vector containing RASSF1C produced a 32-kd protein on sodium dodecyl sulfate–polyacrylamide gels, close to their respective predicted molecular masses of 39 and 32 kd (data not shown). Expression vectors in pcDNA3.1 for mutant and wild-type p53 and their transfection and activity have been described previously *(28)*.

The RASSF1A expression vector was transfected into NSCLC NCI-H1299 cells expressing RASSF1C, but not RASSF1A, by use of Lipofectamine plus (Life Technologies, Inc.) according to the manufacturer's recommendations. For transient transfection studies, approximately 5×10^5 NSCLC NCI-H1299 cells, harvested from 80%–90% confluent cultures in complete medium, were transfected with 1 μg of purified plasmid DNA. Samples were plated in a minimum of triplicate, and cells were collected 48 hours after transfection. Because the pcDNA3.1+ expression vector contains a neomycin resistance gene, clones expressing RASSF1A were selected in complete medium supplemented with G418 (800 μg/mL). Stable clones were maintained in complete medium supplemented with G418 (600 μg/mL). We confirmed that the clones were expressing the transfected RASSF1A gene by isolating total RNA from individual clones and performing RT–PCR as described above. We also transfected NCI-H1299 cells with the vector containing no inserts and isolated stable clones.

The RASSF1A and RASSF1C cDNAs were also cloned in the retroviral vector pBABEpuro and were resequenced to confirm that the genes were in the correct sequence and orientation *(29)*. Virus was prepared in the 293 cell-based Phoenix packaging cell line as described previously *(29)* from cells infected either with the vector alone or with constructs containing the RASSF1A or RASSF1C cDNA. Culture supernatants were collected by centrifugation at 500*g* at 37 °C for 10 minutes and used to infect NSCLC NCI-H1299 cells as described previously *(29)*. Because the viral vector contains a puromycin resistance gene, infected cells were selected with 1 μg/mL of puromycin for 7 days. Cells surviving the selection and containing the transgene were pooled, and total cell extracts were made. Western blot analysis was performed as described previously *(30)* to verify protein expression of the transfected genes. The protein bands were visualized with the Pierce SuperSignal Kit (Pierce Chemical Co., Rockford, IL).

Tumorigenicity Testing

The *in vitro* growth characteristics of NSCLC NCI-H1299 clones that express RASSF1A were tested for anchorage-dependent and anchorage-independent (soft agar) growth. After 48 hours of growth in nonselective medium, transiently transfected NSCLC NCI-H1299 cells were detached with trypsin and diluted, usually 10- to 25-fold, in complete medium containing 800 μg/mL of G418 and plated into fresh 100-mm dishes. The medium was changed twice weekly. After 14 days, the medium was removed, the plates were washed with phosphate-buffered saline (PBS), and the colonies were stained with 1% methylene blue in 50% (vol/vol) ethanol. For the anchorage-independent, soft agar-growth assays, 1000 RASSF1A-expressing cells were suspended and plated in 0.33% Noble agar (Sigma Chemical Co., St. Louis, MO) in complete medium supplemented with 600 μg/mL G418 and layered over a 0.50% agar base in complete medium. After 21 days, colonies greater than 0.2 mm in diameter were counted.

For retrovirally infected cells, anchorage-independent growth assays were performed as follows: 10 000 viable selected cells from each infection were plated in 0.33% soft agar over a 0.50% agar base in Dulbecco's modified Eagle medium (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum. After 21 days, colonies greater than 0.2 mm in diameter were counted.

We also tested the ability of RASSF1A-infected cells to grow *in vivo* in nude mice. Male BALB/c nude (nu/nu) 3- to 6-week-old mice (Charles River Laboratories, Wilmington, DE) were irradiated on day 0 of the experiment in groups of five animals by a 5-minute exposure to 350 cGy from a cesium source. The

next day, each mouse was given an injection subcutaneously on its flank with 0.2 mL of sterile PBS containing 10^7 viable parental, vector control, or RASSF1A retroviral-infected NSCLC NCI-H1299 tumor cells. Mice were monitored every 2–3 days for tumor size; once tumors reached greater than 1500 mm^3, the mice were killed. All animal care was in accord with institutional guidelines.

Antibody Preparation

The entire RASSF1C open reading frame was used to make a glutathione *S*-transferase (GST) fusion protein, which was expressed in *Escherichia coli* by use of an established procedure *(31)*, and was used to make rabbit polyclonal antibodies to be described in detail elsewhere. For the western blot analysis, the antiserum was used at a 1 : 1000 dilution in 5% nonfat milk in PBS. Specificity was determined by western blotting of H1299 cells transfected with vector (negative control) and the various RASSF1-expression constructs (e.g., *see* Fig. 6).

Statistical Analysis

Statistical analysis was performed by use of χ^2 and Fisher's exact tests for differences between groups. Overall survival curves were calculated by use of the Kaplan–Meier method, and survival curves were compared with the log-rank statistic *(32)*. All analyses, including univariate, multivariate, and Cox analyses, were performed by use of SPSS Windows version 9.0.1 (SPSS Inc., Chicago, IL). All statistical tests were two-sided.

Results

Characterization of the RASSF1 Gene

To determine if the RASSF1A gene was mutated in lung and breast cancers, we performed extensive mutational analysis of the RASSF1A isoform with the use of single-strand conformation polymorphism assays on genomic DNA. We had previously found no RASSF1C mutations in 77 lung cancer cell line samples *(7)*. By use of the RASSF1A sequence as a reference, we found several polymorphisms, including the following: codon 21 (AAG to CAG), Lys to Gln; codon 28 (CGT to CGA), no amino acid change; codon 49 (GGC to GGT), no amino acid change; codon 53 (CGC to TGC), Arg to Cys; codon 129 (GAC to GAG), Asp to Glu; codon 133 (GCT to TCT), Ala to Ser; and codon 325 (TAT to TGT), Tyr to Cys.

Expression of RASSF1A and RASSF1C in Lung and Breast Cancer Cell Lines

RASSF1 is located within a region frequently affected by allele loss during growth of lung, breast, head and neck, kidney, and cervical tumors *(1–5)*. We investigated whether RASSF1A and RASSF1C are expressed in lung and breast cancer cell lines. We used isoform-specific RT–PCR to examine the expression of RASSF1A and RASSF1C in lung and breast tumor cell lines and in normal lung and breast epithelial cultures (Fig. 2). RASSF1A was expressed in normal lung epithelial cultures (NHBE and SAE cultures), in a normal breast epithelial culture (Fig. 2, C), but not in 32 (100%) of 32 SCLC lines, in 17 (65%) of 26 NSCLC cell lines, and in 15 (60%) of 25 (60%) breast cancer cell lines. Representative data are shown in Fig. 2. By contrast, RASSF1C was expressed in nearly all of the lung and breast cancer cell lines tested, with the exceptions of several lung and breast cancer lines with known homozygous deletions that include the RASSF1 locus. In resected lung adenocarcinomas, RASSF1A was expressed in only two of five cancers, while RASSF1C was expressed in all cancers (Fig. 2, C).

During RT–PCR analysis for RASSF1A, we frequently noted two closely spaced bands in RASSF1A-expressing tumors and in NHBE cultures (Fig. 2). We sequenced these RT–PCR products and found that the larger band corresponded to RASSF1A, while the smaller product represented a different transcript, RASSF1F (GenBank Accession #AF286217). This transcript skips exon 1C to produce an mRNA encoding a predicted truncated peptide of 92 amino acids ending within the DAG-binding domain (Fig. 1, D). The biologic function, if any, of RASSF1F is unknown. In nearly all of the samples, RASSF1F is expressed when RASSF1A is expressed. However, in some breast cancers and normal breast epithelial cultures (*see* Fig. 2 for examples), RASSF1A is expressed without RASSF1F expression.

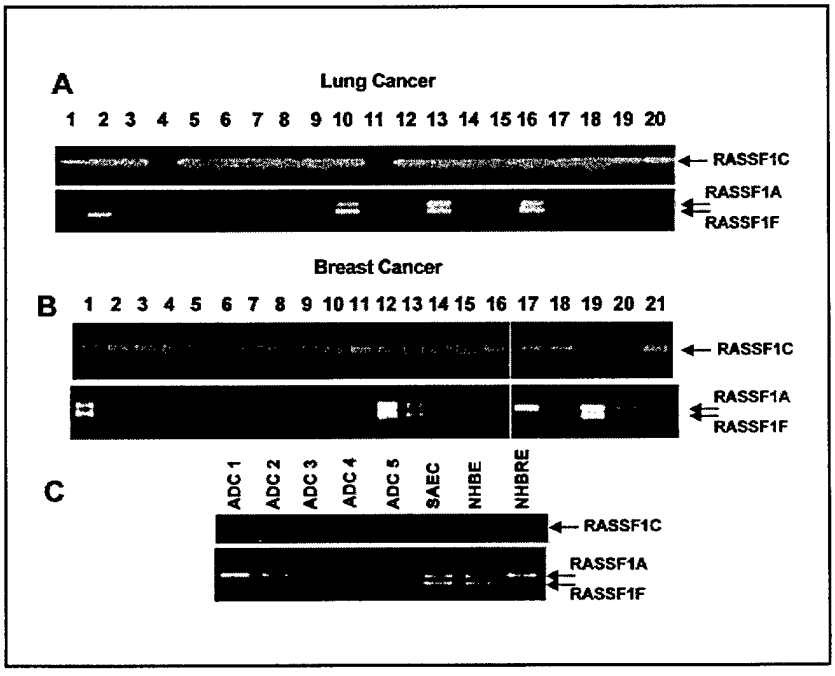

Fig. 2. RASSF1A and RASSF1C messenger RNA levels detected by isoform-specific reverse transcription–polymerase chain reaction (RT–PCR) in a sampling of lung cancer cell lines (**A**), breast cancer lines (**B**), and resected lung tumors and normal human lung and breast epithelial cultures (**C**). All RT–PCR products were separated on 2% agarose gels and were identified by staining with ethidium bromide. **Arrows** indicate location of transcripts. **A**) Lung cancer lines tested in **lanes: 1** = H157; **2** = H358; **3** = H727; **4** = H740; **5** = H748; **6** = H838; **7** = H1184; **8** = H1299; **9** = H1304; **10** = H1437; **11** = H1450; **12** = H1770; **13** = H1792; **14** = H1963; **15** = H1993; **16** = H2009; **17** = H2077; **18** = H2108; **19** = HHCC44; and **20** = HCC78. **B**) Breast cancer lines tested in **lanes: 1** = HCC38; **2** = HCC1187; **3** = HTB19; **4** = HTB20; **5** = HTB22; **6** = HTB23; **7** = HTB24; **8** = HTB25; **9** = HTB26; **10** = HTB27; **11** = HTB121; **12** = HTB129; **13** = HTB130; **14** = HTB131; **15** = HTB132; **16** = HTB133; **17** = HCC1395; **18** = HCC1428; **19** = HCC1569; **20** = HCC1806; and **21** = HCC2157. **C**) Resected lung adenocarcinoma samples (ADC 1–5) and cultures of normal small-airway epithelial cells (SAECs), normal human bronchial epithelial (NHBE) cultures, and normal human breast epithelial (NHBRE) cultures.

Methylation Status of the RASSF1A Promoter Region

Aberrant promoter methylation in tumors has been found to lead to the loss of gene expression of several tumor suppressor genes in human cancers *(14)*. To assess whether the loss of RASSF1A expression in lung cancer was the result of promoter hypermethylation, we determined the CpG methylation status in the 5′ region of RASSF1A (from –800 to +600 bp of the predicted RASSF1A transcript start site) by sequencing sodium bisulfite-modified DNA from eight lung cancer cell lines. All of the six lung cancer cell lines not expressing RASSF1A exhibited methylation of almost all CpG dinucleotide sites in the putative promoter region (data available online at the Journal website). The two lung cancer cell lines that did express RASSF1A either were not methylated at these CpG sites or showed limited methylation. By contrast, no methylation was found in CpG sites in the presumed RASSF1C promoter region of these eight cell lines.

To confirm that promoter hypermethylation contributes to the lack of expression of RASSF1A in the lung cancer cell lines, we assessed the effect of 5-aza-2′-deoxycytidine, a drug that inhibits its DNA methylase, on RASSF1A expression. We exposed the RASSF1A-nonexpressing NSCLC line NCI-H157 to 5-aza-2′-deoxycytidine and found re-expression of RASSF1A by this cell line but little or no change in the expression of the housekeeping gene GAPDH or in the expression of RASSF1C (Fig. 3).

Methylation-Specific PCR Analysis of the Promoter Region of RASSF1A in Lung and Breast Cancers

To determine the methylation status of the promoter region of RASSF1A in primary lung and breast cancers, we used methylation-specific PCR analysis. Genomic DNA from a large number of primary resected NSCLCs, paired lung tissues resected from the same patients but not involved with the cancer, primary resected breast cancers, and a large panel of lung and breast cancer cell lines were treated with sodium bisulfite and tested for the presence of methylated and unmethylated CpG dinucleotides in the promoter region of RASSF1A (Fig. 4). All of the primary resected NSCLCs and non-tumor-paired samples contained unmethylated promoter sequences, which were expected because these resected tumors were not microdissected and were contaminated with stromal cells. However, 32 (30%) of 107 primary NSCLCs, 47 (100%) of 47 SCLC lines, and 19 (49%) of 39 primary breast cancers exhibited the methylated RASSF1A allele (Fig. 4; Table 1). By contrast, no methylated alleles were detected in 104 paired resected nonmalignant lung tissues (Fig. 4; Table 1).

We found a high frequency of methylated RASSF1A alleles in the panel of lung and breast cell cancer lines (Table 1). Because the lung and breast cancer cell lines represent essentially clonal populations of cancer cells without contaminating normal cells, we tabulated the frequency of the methylated and unmethylated RASSF1A alleles (Table 2). While the lung and breast cancer lines often derive from clinically more aggressive lesions than the average population of tumors *(19–21)*, our previous studies *(20,21)* have shown that cancer cell lines continue to retain the genetic alterations found in the uncultured cancer specimens from which they were derived. The presence of only the methylated allele is consistent with either the methylation of both parental alleles or the retention of the methylated allele and the loss of the unmethylated 3p allele. All of the SCLC cell lines

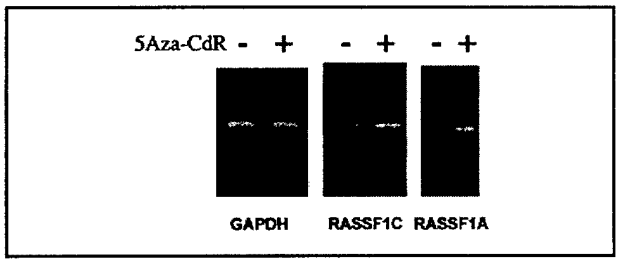

Fig. 3. Expression of RASSF1A after treatment of lung cancer cells with 5-aza-2′-deoxycytidine (5Aza-CdR). NCI-H157, a non-small-cell lung carcinoma (NSCLC) cell line that expresses RASSF1C but not RASSF1A, was grown in the presence (+ lanes) and absence (– lanes) of 0.5 μ*M* 5Aza-CdR for 48 hours. Total RNA was isolated, complementary DNA was prepared, and isoform-specific reverse transcription–polymerase chain reaction was performed for RASSF1A, RASSF1C, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control.

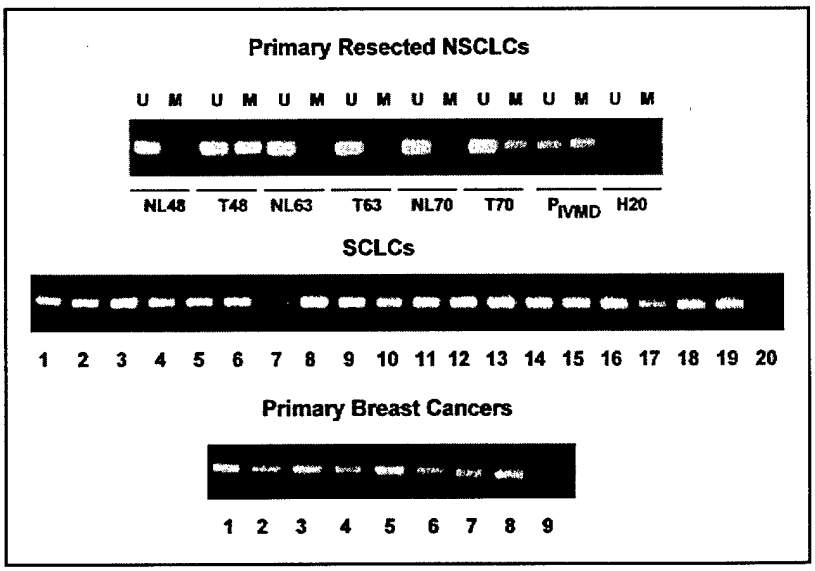

Fig. 4. Methylation-specific polymerase chain reaction (PCR) for the detection of methylated RASSF1A 5′ CpG sequences in primary resected non-small-cell lung carcinomas (NSCLCs) and their accompanying normal lung tissue (**upper panel**), small-cell lung carcinoma (SCLC) cell lines (**middle panel**), and primary breast cancers (**lower panel**). Representative samples are shown. For resected NSCLCs, U = results with primers specific for unmethylated sequences; M = results with primers specific for methylated sequences. NL = normal lung tissue; T = tumor; P = results with peripheral blood lymphocyte DNA, which is unmethylated or *in vitro* methylated (IVMD); and H20 = negative controls with water blanks. For SCLCs, **each lane** shows the PCR results for the methylated sequences from a different cell line. **Lane 20** is negative control. For the breast cancers, **each lane** shows the PCR results for methylated sequences from a different sample. PCR products were separated on 2% agarose gels, and bands were detected after staining with ethidium bromide.

Table 1. Frequency of methylation-specific polymerase chain reaction assay for detection of RASSF1A CpG island-methylated alleles in lung and breast cancers

DNA sample source*	No. tested	No. of methylation alleles (positive) (%)
Primary resected NSCLCs	107	32 (30%)
Corresponding nonmalignant lung	104	0 (0%)
NSCLC lines	27	17 (63%)
SCLC lines	47	47 (100%)
Primary resected breast cancers	39	19 (49%)
Breast cancer lines	22	14 (64%)

*NSCLC = non-small-cell lung carcinoma; SCLC = small-cell lung carcinoma.

Table 2. Presence of methylated and unmethylated RASSF1A alleles in 97 lung and breast cancer cell lines*

RASSF1A CpG genotype					
Methylated allele	Unmethylated allele	SCLC	NSCLC	BCCL	Total
+	+	0	4	4	8
+	−	47	13	10	70
−	+	0	10	7	17
−	−	1	0	1	2†
Total		48	27	22	97

*SCLC = small-cell lung cancer; NSCLC = non-small-cell lung cancer; BCCL = breast cancer cell lines.

†The two tumor cell lines with methylation-specific polymerase chain reaction genotypes lacking both methylated and unmethylated alleles (SCLC line NCI-H740 and breast cancer line HCC1500) were known to have homozygous deletions including the RASSF1 locus in chromosome region 3p21.3.

showed only the methylated allele or lacked RASSF1A entirely because of a homozygous deletion, consistent with the nearly universal 3p21.3 allele loss in SCLC *(1,20,33)*. Of the NSCLC cell lines, 13 (48%) of 27 (Table 2) had only the methylated RASSF1A allele, and 10 (37%) of 27 had only the unmethylated allele, consistent with a lower rate of 3p21.3 allele loss in this tumor type *(1)*. Likewise, 10 (45%) of 22 samples (Table 2) of breast cancer cell lines had only the methylated allele, and seven (32%) of 22 had only the unmethylated allele, again consistent with the rate of 3p21.3 allele loss found in breast cancer *(21)*. As expected, two tumor lines shown previously to have homozygous deletions involving the 3p21.3 region were negative for both the methylated and the unmethylated allele (Table 2) *(7,8)*.

For a subset of 61 lung and breast cancer cell lines, we performed both expression and methylation analysis and found a statistically significant association ($P<.001$, Fisher's exact test) between the presence of methylated RASSF1A alleles and the loss of RASSF1A expression. In 12 samples, RASSF1A was expressed in the absence of a methylated allele; in 44 samples, RASSF1A was not expressed in the presence of a methylated allele; in four samples, RASSF1A was not expressed in the absence of methylated allele (presumably because of some other inactivating mechanism); and in one sample (a breast cancer cell line), RASSF1A was expressed in the presence of both a methylated and an unmethylated allele. These data show the critical association of RASSF1A methylation with loss of RASSF1A expression.

We next assessed whether there was any association between RASSF1A promoter methylation and clinical findings in the patients with primary NSCLC. We found no statistically significant association between RASSF1A methylation and age, sex, tumor–node–metastasis (TNM) pathologic stage *(18)*, or tumor histology in 107 resected NSCLCs (data not shown). In addition, we found no statistically significant association between RASSF1A methylation and age, TNM pathologic stage, tumor histology, estrogen or progesterone receptor status, or HER2/Neu expression in 39 primary resected breast cancers (data not shown).

Survival among lung cancer patients differed by the methylation status of RASSF1A ($P = .046$) (Fig. 5). Also, by univariate analysis, in this group of 107 patients with NSCLC treated with an attempt at curative surgical resection, tumor (T1, T2, and T3), lymph node stage (N1 and N2), and reported weight loss were statistically significant predictors of adverse survival. Neither smoking history (yes/no or pack-years with 40 pack-year cutoff) nor treatment differences (all patients had surgical resection of lobectomy or pneumonectomy, and only five had prior radiotherapy or chemotherapy) accounted for the adverse survival. Because a multivariate analysis is of limited use with a small sample size, we performed a Cox proportional hazards regression analysis by use of RASSF1A methylation and the main univariate factors (tumor, lymph node stage, and weight loss). RASSF1A methylation was not found to be an independent prognostic factor of survival. However, this result could be due to small numbers because even lymph node stage (a known prognostic factor) was also no longer an independent factor in the analysis. Currently, we are studying a much larger cohort of NSCLC patients to determine whether RASSF1A methylation is an independent prognostic factor of survival.

Effect of Exogenous Expression of RASSF1A on Tumor Cell Phenotype

We examined the effect of RASSF1A on the tumor cell phenotype by three methods. We used anchorage-dependent colony

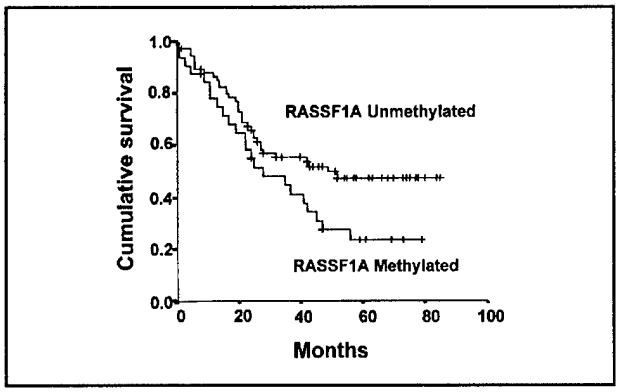

Fig. 5. Kaplan–Meier survival curve for 107 patients with resected non-small-cell lung carcinomas based on RASSF1A methylation status (32 methylated and 75 not methylated). For the patients with unmethylated RASSF1A alleles, the number of cases = 75, censored = 39, and events = 36, with a mean overall survival of 52 months (95% confidence interval [CI] = 44 to 59) and a median overall survival of 49 months (95% CI = 44 to 59); for the patients with methylated RASSF1A alleles, the number of cases = 32, censored = nine, and events = 23, with a mean overall survival of 37 months (95% CI = 27 to 46) and a median overall survival of 28 months (95% CI = 9 to 47). The log-rank test statistic for equality of survival distributions for RASSF1A methylation was 3.97, with df 1, $P = .0463$. The patients at risk for each group were: RASSF1A unmethylated—12 months (n = 63), 36 months (n = 34), and 60 months (n = 16); RASSF1A methylated—12 months (n = 24), 36 months (n = 13), and 60 months (n = 5).

formation as a measure of proliferation and anchorage-independent colony formation as a measure of malignant potential. We also directly assessed *in vivo* tumor formation.

We first cloned RASSF1A cDNA into pcDNA3.1+, an expression vector that contains a selectable marker, and transfected NCI-H1299 cells, which lack endogenous RASSF1A expression. After selection for 14–21 days, we determined colony formation of NCI-H1299 cells in both anchorage-dependent and anchorage-independent assays. Expression of RASSF1A in NCI-H1299 cells resulted in a 40%–60% decrease in anchorage-dependent colony formation and in an approximate 90% decrease in anchorage-independent colony formation compared with cells transfected with the pcDNA3.1 vector alone (Fig. 6, A). Because NCI-H1299 cells have an intragenic p53 homozygous deletion *(34)*, transient expression of wild-type p53 can serve as a positive control for growth inhibition. Indeed, expression of wild-type p53 in NCI-H1299 cells resulted in a 80% and 95% reduction in colony formation in anchorage-dependent and anchorage-independent assays, respectively (Fig. 6, A). Several clones of NCI-H1299 cells transfected with RASSF1A were isolated in selective medium and were found to express RASSF1A by northern blot analysis (Fig. 6, B). Although the clones grew well *in vitro*, each had reduced anchorage-independent colony formation by approximately 90% compared with the vector-transfected control clones (Fig. 6, C).

To eliminate the possibility that the pcDNA3.1+ vector me-

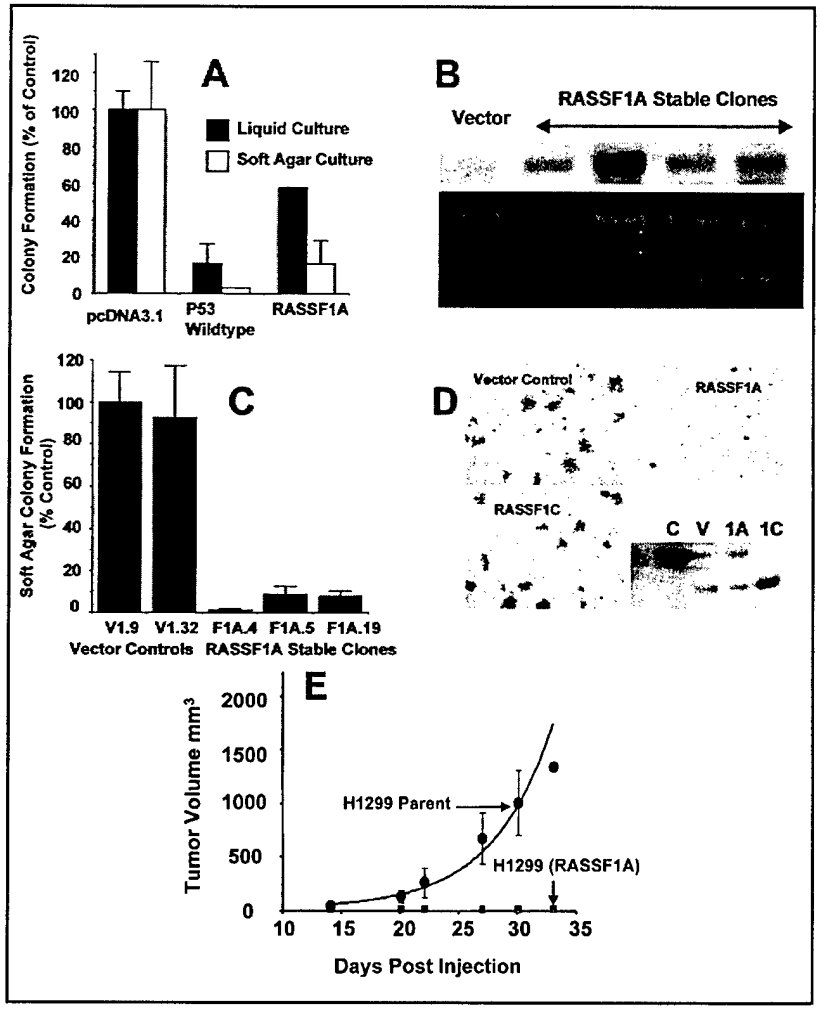

Fig. 6. Effect of RASSF1A on the *in vitro* and *in vivo* growth of the non-small-cell lung carcinoma (NSCLC) cell line NCI-H1299. **A)** Anchorage-dependent and anchorage-independent colony formation after transfection of NCI-H1299 cells with the empty vector (pcDNA3.1+) or pcDNA3.1+ expression vectors containing wild-type p53 or RASSF1A. For analysis of anchorage-dependent growth, after 2 days in nonselective growth medium, transfected NCI-H1299 cells were diluted into 100-mm^2 dishes with selective medium. Transfected cells were plated in liquid medium (for anchorage-dependent assays) or soft agar (for anchorage-independent assays) containing 800 μg/mL of G418. Colonies were stained with methylene blue in anchorage-dependent experiments after 14 days. Results represent the average of eight to 12 experiments in liquid medium and three soft-agar experiments. Standard deviations are shown or are less than 2%. **Solid bars** = anchorage-dependent growth (95% confidence interval [CI] = 0 to 36 for wt-p53 (wild-type) and 52 to 60 for RASSF1A); **open bars** = anchorage-independent growth (95% CI = 0 to 6 for wild-type (wt)-p53 and 0 to 39 for RASSF1A). **B)** Northern blot analysis of the RASSF1A expression in stable clones of NCI-H1299 cells transfected with the pcDNA3.1+ vector or pcDNA3.1+ containing RASSF1A complementary DNA (cDNA). The vector control (vector) and four separate clones with various RASSF1A messenger RNA levels are shown. Several of these clones were used in the anchorage-independent growth assay shown in **D.** Ethidium bromide staining of the ribosomal RNA is shown as a loading control. The clones were also verified to express the RASSF1A isoform by reverse transcription–polymerase chain reaction with the use of isoform-specific primers (data not shown). **C)** Soft-agar (anchorage-independent) colony formation in stable clones of NCI-H1299 cells transfected with the pcDNA3.1+ vector or pcDNA3.1+ containing RASSF1A cDNA. The means and standard deviations are shown. For each of the RASSF1A-expressing clones, the 95% CI = 0 to 4 for F1A.4, 2 to 16 for F1A.5, and 3 to 14 for F1A.19. **D)** NCI-H1299 cells were infected with the pBABEpuro retrovirus expression vectors containing either the vector control or the RASSF1A or RASSF1C cDNAs. Infected cells (10 000 per plate) were suspended in 0.33% agar, and the suspension was layered over a 0.5% agar base. Colonies greater than 0.2 mm in diameter were counted after 21 days. The **lower right panel** shows a representative western blot, developed with a rabbit antibody to the RASSF1-glutathione *S*-transferase fusion protein, to verify the expression of the RASSF1 proteins. C = positive control generated by transient transfection of NCI-H1299 cells with pcDNA3.1+ containing RASSF1A cDNA; V = infection of NCI-H1299 cells with the retroviral vector control (note runover from positive control); 1A = infection of NCI-H1299 cells with the retroviral vector containing RASSF1A; and 1C = infection of NCI-H1299 cells with the retroviral vector containing RASSF1C. **E)** Effect of RASSF1A on the *in vivo* growth of NCI-H1299 cells. Approximately 10^7 viable NCI-H1299 cells expressing RASSF1A were injected into the flanks of each of five previously irradiated BALB/c (nu/nu) nude mice. Tumor size was monitored over time, and size is shown in cubic millimeters. The average volume of tumors grown in more than 20 mice that were given an injection of vector-transfected NCI-H1299 cells is shown (H1299 parent). Mice that were given an injection of RASSF1A-infected NCI-H1299 cells grew no measurable tumors.

diated the growth-suppression effects, we infected NCI-H1299 cells with retroviral-expression vectors containing RASSF1A or RASSF1C and tested the ability of these cells to grow in an anchorage-independent manner. Cells expressing RASSF1A had a marked reduction in the ability to form soft-agar colonies compared with cells infected with the retroviral empty vector or the retroviral vector containing RASSF1C (Fig. 6, D). Cells expressing the retroviral vector formed 3200 colonies per 10000 cells plated. RASSF1A-expressing cells formed only 19% of the vector control colonies, while RASSF1C formed 108% of the vector control. RASSF1A- and RASSF1C-infected cells grew well *in vitro* and showed no signs of toxicity or apoptosis (data not shown).

Finally, we tested the ability of the retrovirally infected NCI-H1299 cells to form tumors in nude mice. Cells transfected with the vector (parental cells) formed tumors rapidly (Fig. 6, E). By contrast, cells infected with RASSF1A retroviral vector and expressing the RASSF1A protein had much lower tumorigenicity *in vivo* (Fig. 6, E).

Discussion

We have found strong evidence that RASSF1A, but not RASSF1C, functions as a tumor suppressor gene that undergoes epigenetic inactivation in cancers by methylation of the CpG islands in the promoter region. Whereas normal lung and breast epithelial cells expressed both RASSF1A and RASSF1C, many lung and breast cancer cell lines did not express RASSF1A, although they did express RASSF1C. These tumor cell lines and uncultured primary lung and breast cancers frequently acquired RASSF1A 5′ CpG island hypermethylation, which was not found in paired lung tissues not involved with cancer from the same patient. Exposure of an NSCLC line to the methylase inhibitor 5-aza-2′-deoxycytidine restored expression of RASSF1A. We found that the loss of RASSF1A expression in a sample of resected NSCLCs was associated with decreased patient survival. Ectopic expression of RASSF1A by transfection by use of several different vectors into a cell line devoid of endogenous RASSF1A suppressed anchorage-independent growth (a measure of metastatic potential) and tumor formation in nude mice. Furthermore, although there was no evidence of *in vitro* morphologic changes, RASSF1A suppressed proliferation in an anchorage-dependent colony-formation assay. Independently, Dammann et al. *(15)* have recently reported similar results.

There is mounting evidence that tumor suppressor genes can be inactivated by tumor-acquired methylation of their promoter regions; indeed, this method of tumor suppressor gene inactivation may be more common than amino acid sequence-altering mutations *(14)*. We found only the methylated RASSF1A allele in 45%–100% of the tumor cell lines, depending on the tumor type, which was consistent with either methylation of both parental alleles or loss of the unmethylated allele. Because the 3p21.3 region, where RASSF1 is located, undergoes frequent allele loss in a variety of human tumors, including those of the head and neck, kidney, and cervix *(5)*, it will be important to extend the RASSF1A studies to these types of cancers. Although the methylation studies were prompted by the fact that we did not find any tumor-acquired, amino acid sequence-altering mutations in either RASSF1A or RASSF1C in our earlier study *(7)*, in this study, we did identify several polymorphisms in the RASSF1A-coding region of six NSCLC cell lines, several of which altered the amino acid sequence. Studies are in progress to determine whether any of these polymorphisms have functional consequences.

Analysis of the protein sequence of the RASSF1 isoforms revealed several domains that may aid in identification of specific cellular functions. The presence of a Ras-association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. If so, the observation that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras-effector pathways that stimulate proliferation. Ras mutations rarely occur in SCLC or in breast cancer and are found in only approximately 30% of NSCLCs (usually in adenocarcinomas) *(2)*. Thus, the observation of RASSF1A methylation with the associated loss of expression in many tumors without Ras mutations suggests that inactivation of RASSF1A expression may be a tumorigenic mechanism that is distinct from the production of Ras mutations that lead to the activation of Ras signaling in tumors. However, it is important to note that, although many proteins have been identified that contain Ras-association domain motifs by database analysis *(9)*, the majority of these proteins have not been validated as *bona fide* Ras interactors. Therefore, studies are presently under way to assess the role of RASSF1 in Ras-dependent growth control.

Additional clues to the function of RASSF1A may be found in its protein structure. Kim et al. *(13)* found that both RASSF1A and RASSF1C possess a putative ATM kinase phosphorylation site in their common exon (Fig. 1), based on *in vitro* phosphorylation studies. Dammann et al. *(15)* isolated RASSF1 transcripts by use of a yeast two-hybrid assay, with the DNA repair protein xeroderma pigmentosum A as bait. These findings suggest that RASSF1 products may participate in the DNA damage response or in DNA damage-induced regulation of other cellular signaling events. The presence of a putative DAG-binding domain in RASSF1A but not in RASSF1C suggests studies to test the role of tumor promoters that interact with the RASSF1A isoform in a novel light: Tumor promoters act on proteins with DAG-binding domains by facilitating their movement to the cell membrane, thus allowing them to interact with the cell's signaling components. Thus, tumor promoters such as phorbol esters might be expected to move RASSF1A to the membrane, where it may act in its normal function as a growth suppressor until RASSF1A expression is lost.

Our previous work *(1,3,4,33)* has shown that 3p21.3 allele loss occurs early in lung cancer pathogenesis. Another study *(35)* has shown that promoter methylation of other tumor suppressor genes (e.g., for $p16^{Ink4A}$) can be detected in preneoplastic lung tissues or in histologically noninvolved lung tissue. RASSF1A promoter methylation may also represent a potentially important marker for the development of invasive lung and breast cancers. Because many smokers have genetic alterations in their respiratory epithelium as a result of damage by tobacco carcinogens *(3,4,33,36)*, the discovery of a marker such as RASSF1A promoter methylation may be of great use both for early detection and for prognosis in monitoring chemoprevention efforts. Furthermore, RASSF1A may represent another potential target for pharmacologic re-expression as a novel mode for cancer treatment.

Note added in proof: Five (71%) of seven primary resected uncultured SCLCs were positive for RASSF1A methylation by the methylation-specific PCR.

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Notes

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